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Degree: Master of Science

Year this Degree Granted: 2001

University of Alberta

Fetal Membrane and Placental Prostaglandins in a Murine Model of Parturition

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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Edmonton, Alberta

Fall 2001

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Fetal Membrane and Placental Prostaglandins in a Murine Model of Parturition** submitted by Maria Christine Shallow in partial fulfillment of the requirements for the degree of Master of Science.

ABSTRACT

The synthesis and regulation of prostaglandins (PGs) in murine fetal membranes and placenta in late gestation and at the time of preterm labor (PTL) and term labor (TL) are not known. This study A) measured PGHS-1 and PGHS-2 mRNA expression in late gestation, at TL and at ethanol-induced PTL in these tissues and B) measured PG levels in tissue from control untreated and P_4 treated mice in late gestation and at TL and post-term delivery. PGHS mRNA expression was measured by RNase Protection Assay (RPA) and PGs were measured by Enzyme Immunoassay (EIA). Data were analyzed by one- or two-way ANOVA and Student's t-test, with post-hoc analysis by Tukey's test ($p<0.05$). Tissue PGHS mRNA expression increased in both tissues with TL, but did not change significantly with PTL. Placental $PGF_{2\alpha}$ levels increased prior to TL and were inhibited by maternal P_4 supplementation. In conclusion, murine fetal membrane and placental PGs are important in late gestation, around the time of TL and may be regulated by maternal P_4.

ACKNOWLEDGEMENTS

Thank you to my supervisor, Dr. David Olson, and to my committee, Drs. Sandra Davidge, Peter Mitchell and Larry Guilbert.

Thank you to my labmates for your advice and technical assistance.

Special thanks to my close friends, to Phuong and to Mom, Dad and Susan for all your love, support and encouragement.

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LIST OF ABBREVIATIONS

A.....absorbance

AA..... arachidonic acid

ACTH..... adrenocorticotropic hormone

ANOVA..... analysis of variance

20α-DHP.....20α-dihydroprogesterone

3α-HSD.....3α-hydroxysteroid dehydrogenase

20α-OH-SDH.....20α-hydroxysteroid dehydrogenase

17α-P_4 .....17α-hydroxyprogesterone

17α-P_5 .....17α-hydroxypregnenolone

5α-R1 .....5α-reductase Type 1

ATP..... adenosine triphosphate

3β-HSD.....3β-hydroxysteroid dehydrogenase

BCA ..... bicinchoninic acid

B.....bound

BL .....blank

B_o.....total bound

°C .....degrees Celsius

Ca^{2+}..... calcium ion

cAMP ..... cyclic adenosine monophosphate

CBG ..... corticosteroid binding globulin

COV ..... coefficient of variation

cpm..... counts per minute

CRH ..... corticotrophin releasing hormone

CTP .....cytosine triphosphate

Cu^{2+}.....cupric ion

Cu^{1+}.....cuprous ion

Cx-43 .....connexin-43

DAG.....diacylglycerol

DHEA ..... dehydroepiandrosterone

DNA..... deoxyribonucleic acid

DTT..... dithriothreitol

EDTA.... ethylenediaminetetraacetic acid di-sodium salt

EIA .....enzyme immunoassay

EP..... prostaglandin E receptor

ER ..... endoplasmic reticulum

EtBr..... ethidium bromide

FSH .....follicle-stimulating hormone

FP .....prostaglandin F receptor

g..... gram(s)

GD.....gestational day

GTP.....guanosine triphosphate

h..... hour(s)

[^{3}H].....^{3}hydrogen (tritium)

HPA.....hypothalamo-pituitary-adrenal

ICM .....inner cell mass

i.g. .....intragastrically

ir .....immunoreactive

LDL.....low density lipoprotein

LH ..... luteinizing hormone

LPS.....lipopolysaccharide

M ..... $moles \cdot l^{-1}$

MCR ..... metabolic clearance rate

$MgCl_2$ ..... magnesium chloride

MLCK ..... myosin light chain kinase

min ..... minute(s)

mol ..... moles

mRNA ..... messenger ribonucleic acid

n ..... number of sample

NaCl ..... sodium chloride

ndu ..... normalized denistometric units

NSAID ..... non-steroidal anti-inflammatory drug

NSB ..... non-specific binding

nt ..... nucleotides

P_4 ..... progesterone

P_5 ..... pregnenolone

$[^{32}P]$ ..... ^{32}Phosphorus

PGE_2 ..... prostaglandin E_2

$PGF_{2\alpha}$ ..... prostaglandin $F_{2\alpha}$

PGDH ..... prostaglandin dehydrogenase

PGES ..... prostaglandin E-synthase

PGFS ..... prostaglandin F-synthase

PIPES ..... piperazine-N,N'-bis [2-ethane-sulfonic acid]

PKA ..... protein kinase A

PR ..... progesterone receptor

PREs ..... progesterone response elements

RNA ..... ribonucleic acid

tRNA ..... transfer ribonucleic acid

rpm ..... revolutions per minute

RT ..... room temperature

SDS ..... sodium dodecylsulphate

sec ..... second(s)

SEM ..... standard error of the mean

SHBG ..... sex hormone binding globulin

SPE ..... solid phase extraction

SSH ..... steroid sulfohydrolase

TA ..... total bound

TE ..... tris-EDTA

TTP ..... tyrosine triphosphate

CHAPTER 1. Literature Review

1.1 Introduction

Prostaglandins (PGs) are important mediators of parturition and fetal physiology. Maternal and fetal tissues are intimately associated within the uterus. Therefore, the factors which mediate the processes involved in parturition and fetal intrauterine health can originate from both maternal and fetal tissues. Proper coordination of hormonal signals within the uterus ensures that a pregnancy is carried to term. Disruption in the sequence of events leading to labor at term may result in preterm labor, the rate of which, despite an abundance of research, has not declined in 30 years and continues to be associated with 70-75% of neonatal morbidity and mortality (1). The role of PGs in the process of parturition has been well established (2-7). However, the origin of these PGs and how they are regulated is not clear at term or preterm birth.

Once a PG is synthesized within a cell, it acts either directly on that cell (autocrine) or on neighboring cells (paracrine) (8), and can also act in an endocrine manner (9). Therefore, PGs originating from the fetal membranes and placenta may act on the adjacent myometrium or circulate and have peripheral effects (10, 11), although the half-life of PGs is very short in circulation (12). In the mouse, the contribution of the fetal membranes and placenta to intrauterine PG levels and the regulation of PG synthesis in these tissues are unknown. Therefore, this thesis will focus on the profile of a PG synthetic enzyme in late gestation and at term and ethanol-induced preterm labor in murine fetal membranes and placenta. Additionally, we will determine the effect of maternal progesterone (P_4) administration on PG (PGE_2 and $PGF_{2\alpha}$) levels in these tissues in late gestation and at term labor.

The main focus of the following literature review will be on the role of fetal membrane and placental PGs in the process of parturition. The model used in this study is the mouse because of its importance in genetic manipulation studies, short gestational period (19 days) and cost effectiveness. However, because literature on parturition in sheep is

extensive and knowledge of human parturition is necessary, the pertinent information on intrauterine PG production in these species will also be discussed. This review will begin with an overview of the morphological characteristics and relative orientation of the fetal membranes and placenta. It will continue with a summary of recent literature pertaining to the biochemical characteristics and regulation of enzymatic steps in the PG biosynthetic pathway and the involvement of PGs in parturition and will briefly discuss the role of PGs in fetal physiological processes and placental blood flow. Finally, it will introduce the murine model used in this investigation and overview the objectives of the experiments involved.

1.2 Comparative Placental and Fetal Membrane Morphology

1.2.1 General

The placenta and fetal membranes are derived from embryonic tissue. The placenta is the site of maternal-fetal exchange of nutrients, waste and oxygen (9). The fetal membranes (the amnion and the chorion) surround and protect the fetus, contain the amniotic fluid and are in close contact with the maternal endometrium. Interestingly, the outermost fetal membrane of the rodent may function as an additional site of exchange. As mentioned previously, interaction within and between the intrauterine tissues occurs primarily through paracrine or autocrine cell-cell communication. Additionally, peptide factors such as growth factors, cytokines, PGs, and neurohormones may enter maternal and fetal circulation. These may have an endocrine effect on metabolism, and on the immune and cardiovascular systems (9). In order to understand the ability of the maternal and fetal intrauterine tissues to exchange and respond to hormonal cues, it is necessary to understand their relative positions within the uterus. The development, orientation and function of the fetal membranes and placenta in the human, sheep and rodent will be discussed in this section. The relative orientations of fetal membranes and placenta in each species is depicted in figure 1-1.

1.2.2 Trophoblast

Prior to implantation, the embryo exists as a hollow ball of cells called a blastocyst. The population of cells contained within the blastocyst, the inner cell mass (ICM), develop into the embryo, the amnion, the allantois and the yolk sac. The outermost layer of the

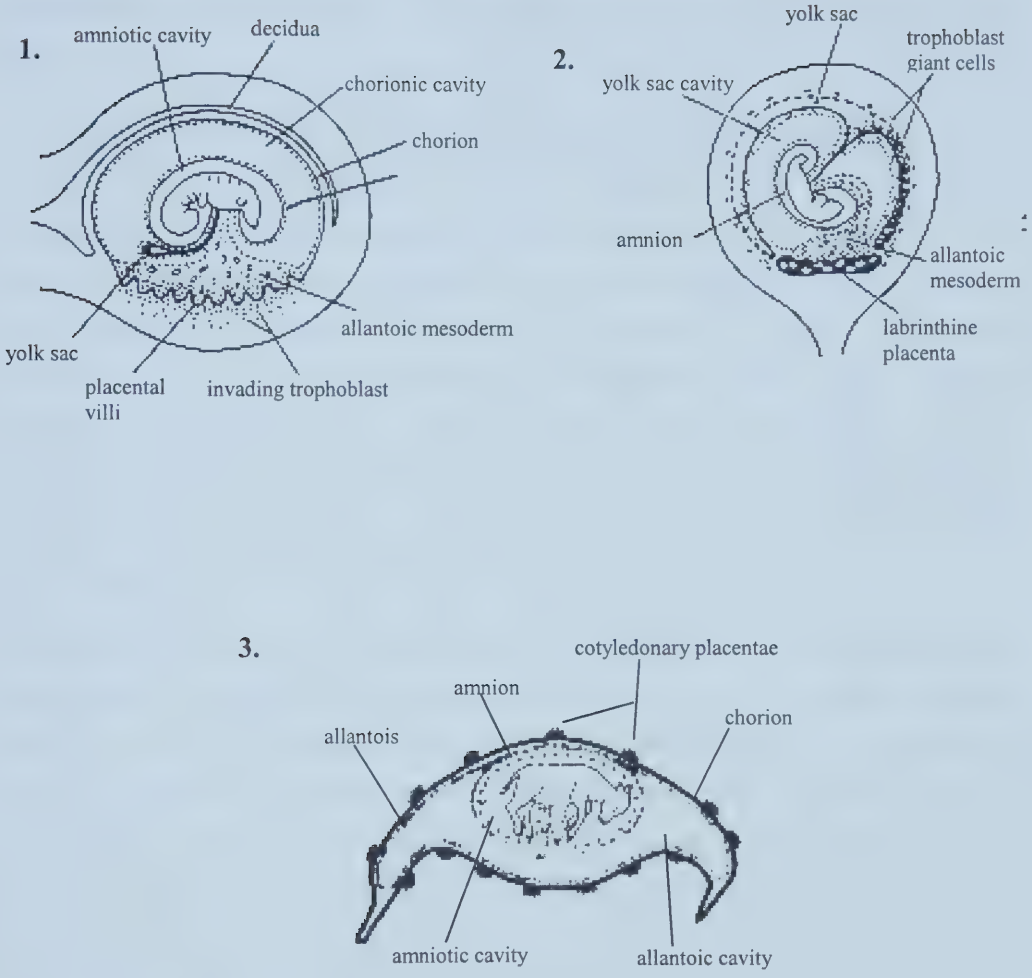

Figure 1-1 Comparative orientation of fetal membrane and placental anatomy

Intrauterine orientation of (*1*) human, (*2*) mouse and (*3*) sheep fetal membranes and placenta(s). See text for description. Illustrations adapted from Pijnenborg, 1981 (13).

blastocyst is composed of trophoblast cells. The region of the outer trophoblast layer that is not adjacent to the ICM differentiate into chorionic trophoblast (or trophoblast giant cells in the rodent) while those cells adjacent to the ICM continue to proliferate and differentiate into two cell layers: the outer differentiated syncytiotrophoblast and the inner proliferative cytotrophoblast. These cell layers develop into the placenta and the chorion (14).

In the human and sheep villous placenta, both the syncytiotrophoblast and the cytotrophoblast form the primary villi (the initial extensions into the maternal endometrium). These become secondary villi when embryonic mesenchyme invades the villi and then tertiary villi when fetal blood vessels (umbilical/allantoic vessels) are formed (described below). Tertiary villi consist of highly differentiated syncytiotrophoblast (surface layer) and the cytotrophoblast (basal layer). With the progression of pregnancy, the two fuse and form a single syncytial layer with some remaining proliferative cytotrophoblast tissue (15). The major route of nutrient supply to the growing fetus is the villous interface. Solute transfer occurs across the syncytium between maternal and fetal blood (16).

Trophoblast invasion varies between species and the pattern of interdigitations formed between fetal trophoblast and maternal endometrium serves to maximize exchange surface area (17). Trophoblasts are invasive, colonizing cells that degrade local extracellular matrix and endocytose the maternal tissue, glands and blood vessels (18). The maternal secretions, blood and tissue particulates serve as nourishment for the developing embryo prior to establishment of placental function (19). In the human (18) and rodent, trophoblasts invade endometrium and expand to form a single organ (discoidal placenta) for maternal-fetal exchange (20-22). In the sheep, a number of small populations of trophoblasts invade the uterine endometrium forming many (approximately 100) small placentomes separated by smooth areas of chorion (17). The remaining trophoblast that does not extend into maternal endometrium to form villi becomes chorion laeve (the outermost fetal membrane – the trophoblast giant cells in the rodent), the marginal zone, the chorionic plate and the basal plate (15).

1.2.3 The Amnion and Chorion

The amnion is derived from the ICM and arises from embryonic ectoderm. It surrounds the fetus as the innermost fetal membrane and contains the amniotic fluid. The chorion laeve is an extension of the chorion around the entire uterine cavity. Except at the placental site, the chorion is in contact with the decidua (in humans) which is immediately next to the myometrium (23). Both the amnion and the chorion develop an underlying mesenchyme and are able to synthesize hormones, such as, oxytocin (OT), corticotropin releasing hormone (CRH), growth factors, cytokines and PGs (9).

1.2.4 The Yolk Sac and the Allantoic Sac

In rodents, the allantoic sac and the yolk sac (vitelline sac) become integrated into the fetal membranes and the placenta. The yolk sac is derived from the embryonic midgut and is partially comprised of fetally-vascularized mesenchyme (vitelline vessels). In humans and sheep, the yolk sac is rudimentary and does not participate in maternal-fetal exchange. In rodents, the yolk sac replaces the chorion and forms the outermost embryonic membrane, the function of which will be discussed below (19).

In all three species, the allantois supplies the vascular network for the placenta (19). Therefore, the placenta is comprised of chorioallantoic tissue. The vascular mesoderm of the allantois fuses with the mesoderm of the chorion. Thus, the allantoic capillaries gain access to the developing fetal portion of the placenta and form the umbilical vessels which carry fetal blood into and from the trophoblast villi or labyrinth structure in rodents. The placenta becomes vascularized with allantoic (umbilical) vessels except where chorionic trophoblast is in contact with maternal blood (17).

1.2.5 Characterization of Placental Morphology

As mentioned previously, the number of sites of trophoblast invasion varies among species and determines the shape and concentration of the placental exchange area. Thus, the single discoidal placenta is characteristic of humans and rodents while the numerous placentomes are characteristic of the sheep. The discoidal placenta represents a highly concentrated area of exchange and the placentomes represent a more dispersed exchange area. Variability in placental structure is categorized based on the following: shape and concentration of exchange area (described earlier), geometrical pattern of interdigitation

of maternal and fetal surfaces, and, kind and number of tissue layers separating maternal and fetal blood (17). These will be discussed below.

The geometrical pattern in which maternal and fetal tissues are spatially arranged to form the placenta can be used to classify placental types. In the human and sheep villous placenta, chorionic branches or villi extend into maternal endometrium and are directly bathed in maternal blood. In contrast, in rodents, the chorion and endometrium fuse more extensively to form the labyrinthine placenta. Within the labyrinth formation, 50% of the lacunae are perfused with maternal blood while the other 50% contain fetal (allantoic/umbilical) capillaries (17).

Classification of placental morphology is also based on the number and type of tissue layers separating maternal and fetal blood. The extent of this endometrial invasion determines the number of layers remaining between fetal and maternal blood. Direct exposure of chorionic tissue to maternal blood is called hemochorial placentation which is characteristic of humans and rodents. This classification can then be further subdivided according to the number of trophoblastic layers. The human placenta then is classified as hemomonochorial, the rodent placenta hemotrichorial and the sheep placenta epitheliochorial (17).

1.2.6 Unique Characteristics of Rodent Placentation

The rodent has two sites where maternal-fetal exchange can occur: the highly vascularized yolk sac and the discoidal chorioallantoic placenta. Brunschwig (1927) was the first to suggest that the yolk sac epithelial membrane may be a site of exchange and that this may be significant for organogenesis (24). The yolk sac is the route of iron transport while the chorioallantoic placenta is the route of calcium transport. Proteins are unable to pass through the chorioallantoic placenta, but can pass through the parietal wall of the yolk sac. The proteins are subsequently taken up by the endodermal cells of the yolk sac visceral wall to pass to the embryo via branches of the vitelline veins lying in the mesenchyme under the visceral endoderm (19).

The yolk sac is the only route for major transport between mother and fetus during the critical stages of organogenesis (before day 10) since the chorioallantoic placenta does not become vascularized by allantoic vessels until day 11 or 12 of gestation (19).

Proteins taken up by visceral yolk sac cells are digested intracellularly and the breakdown products are subsequently released from the cells into fetal circulation. This process has been proposed to be a major source of embryo nutrition and continues once the chorioallantoic placenta has been established. It has been shown that the yolk sac and the chorioallantoic placenta show patterns of increased uptake of some substances and decreased uptake of other substances with advancing gestation. Thus the relative importance of each exchange site changes during gestation – the day 10 embryo cannot survive without the yolk sac while later in gestation, the disruption of the yolk sac is not fatal to the fetus (19).

1.2.7 Decidua

Contact with fetal trophoblast cells induces morphological and endocrinological changes in the maternal endometrium (decidualization), therefore, the endometrium of pregnancy is called the decidua. Anatomically located between the uterine myometrium and the fetal membranes, decidual tissue is secretory and establishes a paracrine relationship between maternal and fetal endocrine systems (25). The human decidua is composed of three sections based on anatomical location: 1) the decidua basalis, which underlies the site of implantation and forms the maternal component of the placenta; 2) the decidua capsularis, which overlies the gestational sac and disappears in late gestation; and 3) the decidua vera (decidua parietalis), which lines the remainder of the uterus and is immediately adjacent to the chorion (9). The process of decidualization is more extensive in humans than in the rodent (26). Human decidua extends to surround the periphery of the chorion while rodent decidua is localized to the area surrounding the site of implantation. The sheep endometrium does not undergo a decidualization reaction (26).

1.2.8 Summary

In summary, knowledge of the orientation and relative position of fetal and maternal tissues within the uterus is important for understanding how endocrine signals for birth can be transmitted *in utero*. The dynamics and role of PG production within the fetal membranes and placenta in relation to the mechanisms leading to the onset of parturition will be discussed in subsequent sections.

1.3.1 Stages of Parturition

The process of parturition has been divided into different phases based on the ability of the myometrium to respond to contractile stimuli (1). Throughout most of pregnancy, the myometrium is in a state of relative quiescence, although it is never without contractile activity (27). This phase, phase 0, is characterized by long duration, low amplitude contractures. Contractures are isolated to regionalized areas of uterine muscle and are, therefore, poorly coordinated (28). Phase 0 is associated with the first 95% of pregnancy. In late pregnancy, the uterus prepares to respond to stimuli that lead to the contractions of labour. It becomes spontaneously active and responsive to exogenous uterotonins. This transition from a quiescent state to a state of readiness for stimulation under the influence of uterotropins is called uterine "activation", corresponding to phase 1 of parturition. Once activation has occurred, the myometrium is able to respond to endogenous or exogenous stimulation by uterotonins such as PGs and OT (29). This response results in the coordinated, high frequency, high amplitude contractions of active labour and corresponds to phase 2 of parturition, also referred to as "stimulation". Postpartum involution corresponds to phase 3. The "initiation" of parturition corresponds to the transition from phase 0 to phase 1 (29). The combination and timing of factors leading to the activation and stimulation of the myometrium is coordinated by fetal and maternal endocrine cues.

1.3.2 The Mechanism of Uterine Contractility

Myometrial contractions are caused by conformational changes in actin and myosin leading to the shortening of the myocyte (3, 30). The conformational changes occur as a result of a series of intracellular mechanisms leading to either the release of calcium from the sarcoplasmic reticulum or increased influx of extracellular calcium. For extensive reviews on this subject see Garfield, 1988, and Garfield, 1990 (31, 32). Briefly, four calcium ions (Ca^{2+}) bind to and activate calmodulin. The Ca^{2+}-calmodulin complex then activates (dephosphorylates) myosin light chain kinase (MLCK). The conformational change in MLCK leads to phosphorylation of the 20 kDa light chains of myosin and causes cross-bridging of the myosin head. This is the means by which factors inhibit or stimulate myometrial contractility. Increasing intracellular Ca^{2+} concentrations promotes

myometrial contractions while decreasing intracellular Ca^{2+} concentrations which decreases the affinity of MLCK for Ca^{2+}-CAM (via cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA)) inhibits myometrial contractions.

1.3.3 Proteins Associated with Parturition

In 1989, Lye and Challis introduced the idea that a number of proteins are upregulated during activation of the myometrium (33) . Considering that these proteins may be upregulated synchronously by the same stimulus, they have been designated a collective term: contraction-associated proteins (CAPs). CAPs include ion channels, which determine resting membrane potential and excitability of the myocytes (34), agonist receptors such as oxytocin receptor (OTR) and prostaglandin $F_{2\alpha}$ receptor (FP) (35) and GAP junctions such as connexin 43 (Cx-43), which connect myocytes and coordinate cell-cell signaling for the synchronous myometrial contractions of active labor (32). This term may be extended to include prostaglandin H-synthase-2 (PGHS-2) as an important contraction associated proteins since it is also upregulated at the same time.

1.4 STEROIDS AND PARTURITION

1.4.1 Progesterone and Estrogen Biosynthesis

In most animal species, pregnancy is maintained by progesterone (P_4). The onset of labor is precipitated by a late gestational decrease in plasma P_4 concentrations and an increase in plasma estradiol (E_2) concentrations (36, 37). The increase in the E:P ratio releases the uterus from the inhibitory effect of P_4 and induces the upregulation of CAPs (described above). Although the present study focuses on the role of maternal P_4 in controlling murine fetal membrane and placental PG synthesis, this discussion will include some information pertaining to E_2 biosynthesis since the ratio of the two is an important consideration. The general steps in steroid biosynthesis are similar in all steroid producing cells (Figure 1-2). P_4 and E_2 are synthesized from stored intracellular cholesterol (stored as esters) or from low-density lipoprotein (LDL)-cholesterol which enters a cell through a specific membrane receptor (38) under the influence of follicle stimulating hormone (FSH) and leutinizing hormone (LH) (39). The human placenta receives LDL-cholesterol from both the fetal and maternal circulation. Cholesterol is metabolized to pregnenolone (P_5) by cholesterol side chain cleavage. P_5 is converted to

P_4 by 3β-hydroxysteroid dehydrogenase (3β-HSD). P_4 can also be synthesized from pregnenolone sulfate (P_5S) by steroid sulfohydrolase (SSH). As well, P_4 can be synthesized from its metabolite 20α-dihydroprogesterone (20α-DHP). Each of these pathways exists in the human placenta (40) and in rat fetal membranes and placenta (41-43). As well, 3β-HSD activity has been detected in human fetal membranes with highest activity in the chorion (44). Both P_5 and P_4, through a series of enzymatic steps, can lead to the production of androgens (androstenedione) which are aromatized to E_2. In the corpus luteum, the theca interna cells contain all of the enzymes necessary for androgen biosynthesis and the granulosa cells are capable of producing progestogens and aromatizing androgens to E_2 (45). Steroidogenic activity (3β-HSD) has also been measured in the rodent placenta both *in vitro* (46, 47) and *in vivo* (41, 42).

In the rodent, P_4 and E_2 are produced in the corpus luteum, which regresses in late gestation under the influence of $PGF_{2\alpha}$ (a process known as luteolysis) (48). Uterine $PGF_{2\alpha}$ upregulates 20α-hydroxysteroid dehydrogenase (20α-OH-SDH) activity (49-51) and induces luteolysis by causing a reduction in luteal blood flow and inducing apoptosis. Ovariectomy in the rodent results in preterm delivery which can be prevented by P_4 supplementation (52). In the mouse, early in gestation, the ovary is the principle source of androgen precursors for E_2 synthesis (45). With increasing gestational age, luteal cell aromatase activity increases (53), but the ability of the luteal cells to convert P_4 to androstenedione decreases (45). At this time, the placenta becomes the primary source of androgen substrate for aromatization to E_2 in the ovary (45).

In sheep, the corpus luteum must be present through the first 50 days of gestation to maintain pregnancy (54). During this time, pregnancy can be maintained in ovariectomized sheep by P_4 supplementation (55). After day 50 of gestation, the placenta becomes the primary site of P_4 production. In the human, P_4 is produced by the corpus luteum until week 5-6 and its removal before this time can result in abortion (56). Luteoplacental shift follows this period as the placenta gradually takes over the role of steroidogenesis and by week 12, the placenta is the primary source of P_4 (40). In the placenta, the syncytiotrophoblast of the chorionic villi is the primary site of steroidogenesis (57).

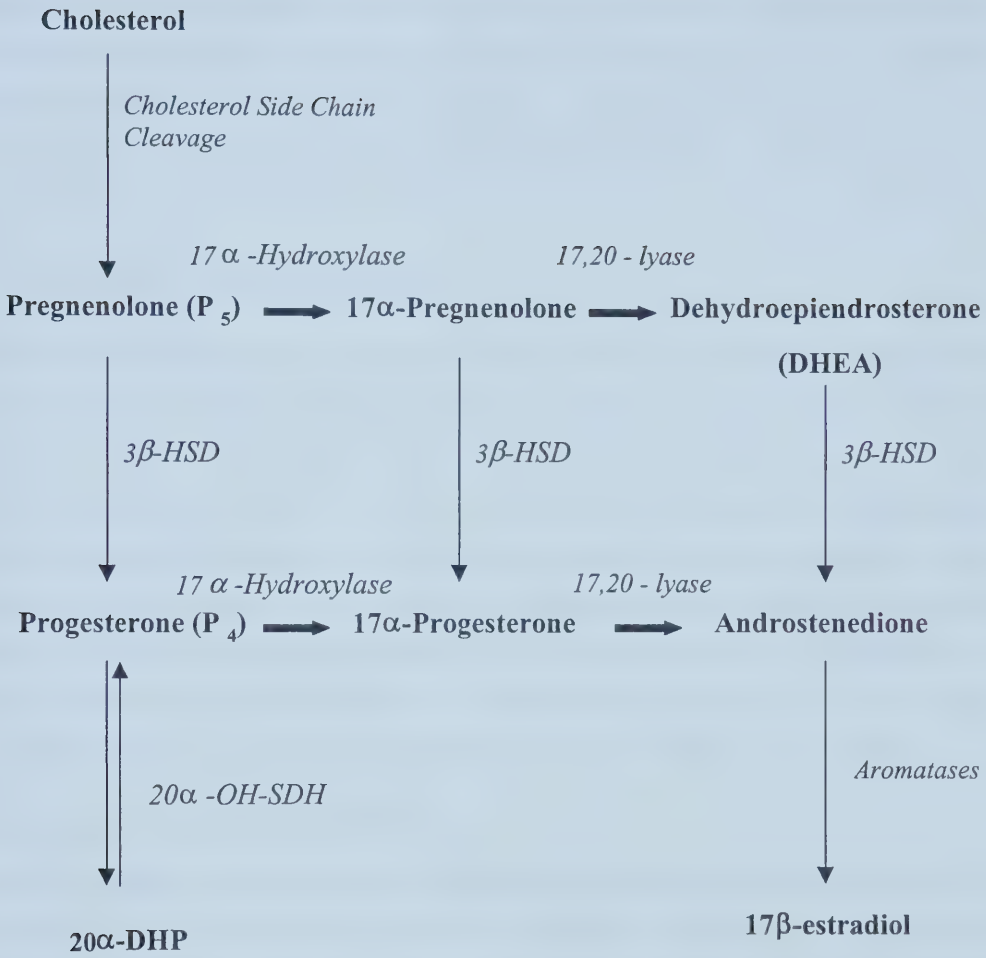

Figure 1-2 Steroid biosynthetic pathway

Once released, P_4 and E_2 are carried in the circulation by corticosteroid-binding globulin (CBG) and sex hormone binding globulin (SHBG), respectively (58). Steroid binding globulins are produced in the liver (59) and more than 95% of steroids secreted into circulation are bound by these proteins (60). The purpose of steroid binding globulins is to prevent rapid degradation of circulating sex steroids, resulting in a prolonged half-life and decreased metabolic clearance rate (MCR). In addition, they act to maintain a dynamic equilibrium between serum binding and target cell activation (61). Plasma levels of SHBG have been shown to correlate with the decrease in P_4 concentrations in late gestation in the rodent (62).

1.4.2 Biological Activity of Progesterone

Progesterone exerts it biological effects by binding first to a cytoplasmic receptor to form a receptor-steroid complex which then undergoes translocation to the nucleus. Within the nucleus P_4 interacts with a nuclear receptor (PR). PR is a member of a group of ligand-activated nuclear transcription regulators. Binding of P_4 to the carboxy-terminal ligand-binding domain of PR causes dimerization of this complex to specific progestin response elements (PREs) in target genes, resulting in modulation of transcription of those genes (63). In general, E_2 induces the expression of PR while P_4 inhibits the expression of PR (64). However, in ovariectomized rats, exogenous administration of P_4 has been shown to maintain PR through rapid and reversible processing of nuclear receptors (65).

1.4.3 Activation of the Fetal HPA Axis

In both humans and sheep, activation of the fetal hypothalamic-pituitary-adrenal (HPA) axis triggers the onset of labour. Activation of the HPA axis changes the steroidogenic capabilities of the placenta. This has been reviewed extensively by Challis (1989), Olson (1995), Mijovic (1996) and Challis (2000) (2, 27, 66, 67). Briefly, in sheep, maturation of fetal HPA function in late gestation is evident by the progressive increases in fetal plasma adrenocorticotropic hormone (ACTH) and cortisol. The initial rise in ACTH precedes the rise in cortisol and cortisol increases exponentially in the last 10 days of gestation with the highest concentrations observed just before term. Fetal cortisol induces increased activity and expression of 17α-hydroxylase. This results in decreased placental P_4 output and increased E_2 synthesis. The rise in E_2 production stimulates an

increase in both placental and uterine PGHS-2 mRNA expression (leading to increased PG synthesis) and in uterine CAPs expression. PGE_2 production by the ovine placenta also provides a positive feedback stimulus to the fetal HPA axis to maintain cortisol secretion by the fetal adrenal Although human labor is not precipitated by a rise in the E:P ratio, the human also has a feto-placental unit of E_2 production. The key difference is that the human placenta does not contain the 17α-hydroxylase enzyme. Rather, the fetus provides the placenta with the C19 E_2 precursors. P_5 is synthesized in the fetal zone of the adrenal from circulating fetal LDL cholesterol and converted in the adrenal fetal zone to dehydroepiandrosterone sulfate (DHEA-S). An hydroxyl moiety is attached by 16-hydroxylase in the fetal liver and the resultant 16OH-DHEA-S is transported to the placenta where it is aromatized to E_2. A mechanism similar to those described above has not been shown in the mouse.

1.5 Characteristics of the Prostaglandin Biosynthetic Pathway

PGs are synthesized from arachidonic acid (5,8,11,14-eicosatetraenoic acid; AA) (Figure 1-3). In general, a given cell type synthesizes only one type of prostaglandin as its major product (8). The biochemical characteristics of the enzymatic reactions involved in this pathway will be discussed below in relation to their regulation in late gestation in the fetal membranes and placenta.

1.5.1 Phospholipases

AA is released from phospholipids by specific acyl hydrolases called phospholipases (PL). This discussion will focus on PLA_2 and PLC. Once released, AA is either re-acylated or oxygenated (e.g. by PGHS) into biologically active metabolites (eicosanoids) such as PGs. The PLA_2 enzymes are a ubiquitous family of esterases that hydrolyze the *sn*-2 acyl ester bond of 1,2-diacyl-sn-3 phosphoglycerides (for a complete review see Rice, 1996 (68). AA is esterified preferentially in the *sn*-2 position of phospholipids. Therefore, the action of PLA_2 results in the release of AA. PLA_2 exists as two isoforms, which are both Ca^{2+} sensitive. The secreted PLA_2 ($sPLA_2$) isoform has a low molecular weight (14 kDa), requires high intracellular Ca^{2+} concentrations for maximal activity (69) and has been classified into four subtypes (I-IV) (70). $sPLA_2$ is secreted and acts on the

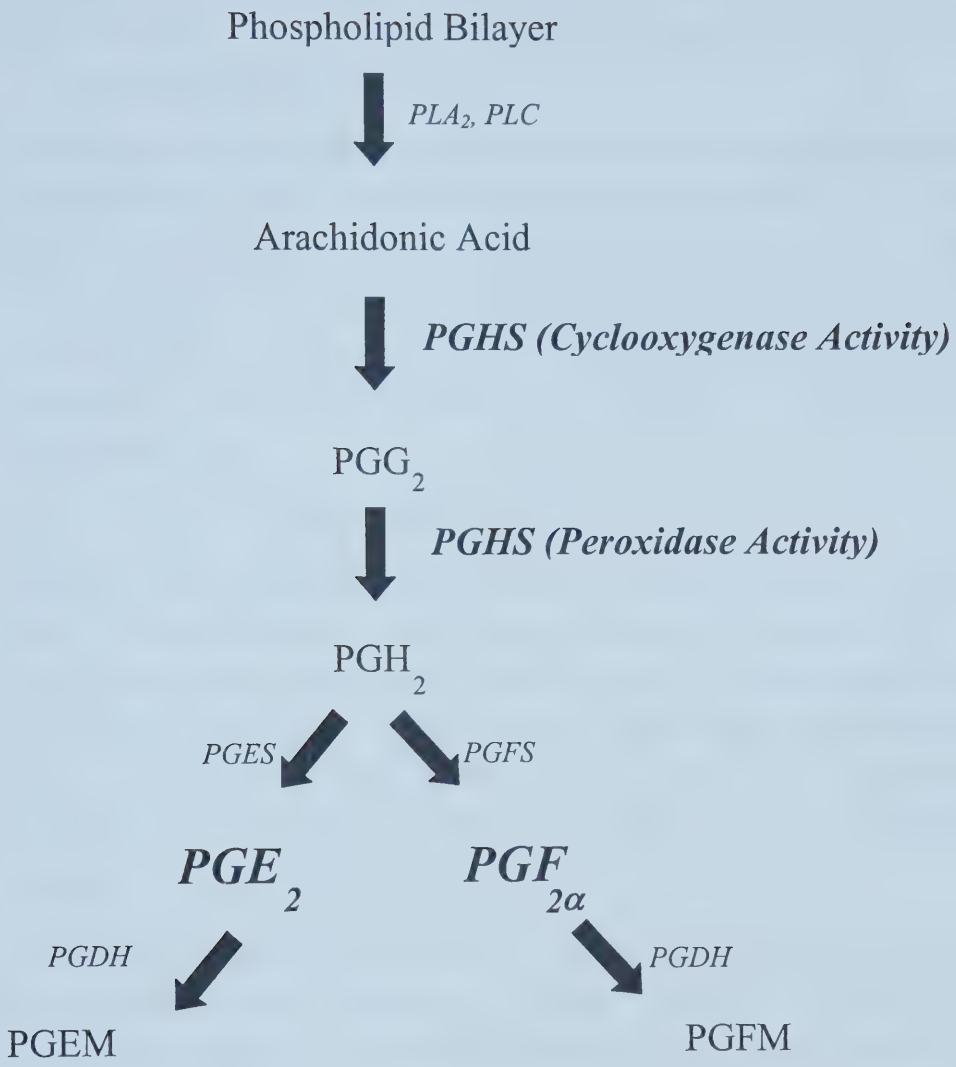

Figure 1-3 Prostaglandin biosynthetic pathway

extracellular surface of the plasma membrane. The second isoform, the high molecular weight (85-110 kDa) cytosolic PLA_2 ($cPLA_2$) is a soluble protein that is Ca^{2+} sensitive,

requiring low μM intracellular Ca^{2+} concentrations for optimal activity (71-73). $cPLA_2$ acts intracellularly and undergoes Ca^{2+}-dependent translocation between the cytoplasm and the plasma membrane (71, 72).

PLC catalyzes the hydrolysis of the phosphodiester bond between diacylglycerol (DAG) and the phosphate moiety of the head group of phospholipids (74). The activation of phosphatidylinositol-specific PLC results in the release of DAG and inositol phosphates. DAG is further metabolized in tissues such as human fetal membranes and decidua to yield glycerols and fatty acids such as AA, which may then serve as a prostaglandin precursor (75). Maximal activity of PLC occurs in presence of high intracellular Ca^{2+} concentrations (75).

1.5.1.1 Phospholipases and Labor

In humans, $sPLA_2$ isozymes have been localized to the fetal membranes (70) and PLA_2 activity has been measured in both the fetal membranes and placenta (76-78). In the human placenta, $sPLA_2$ gene expression has been shown to increase at term labor (79). In the human amnion, PLA_2 enzymatic activity increases significantly in late gestation but no increase has been demonstrated with term labor (76, 80) despite increased amniotic fluid AA concentrations at this time (81). This may indicate that there are other sources of AA, such as fetal lung surfactant (82, 83).

In the sheep, $cPLA_2$ activity is enhanced with term labor in the endometrium but not in the fetal membranes (84). In the mouse, mutations in Type II $sPLA_2$ gene does not alter in the onset or progression of labor (85). Alternately, mice deficient in the $cPLA_2$ gene exhibit delayed labor initiation (delivered on day 22.3) and increased neonatal mortality due to the prolonged gestation (86). This indicates that $cPLA_2$ is essential for murine labor although localization of the enzyme in late gestation and at term labor is unknown in this species.

Information pertaining to the role of PLC in intrauterine AA mobilization is not abundant. PLC activity has been shown to be high in human amnion, chorion and decidua (87, 88). In addition, PLC activity increases in human amnion and decidua (not in chorion), in late gestation (89), but, does not increase with term spontaneous labor (87). The agonists and intracellular mechanisms responsible for the increase and

maintenance of PLC levels are unknown, and do not appear to regulate PLC during parturition.

1.5.2 Prostaglandin H-Synthase

Prostaglandin H-synthase (PGHS) metabolism of AA is considered the rate-limiting step in synthesis of PGs. Risbridger *et al.* (1985) investigated whether AA availability determined the PG synthetic capacity of ovine fetal cotyledonary tissue in late gestation (90). Incubation of trophoblast cells with AA (40uM) had no effect on PG output throughout most of gestation. PG synthesis (both PGE_2 and $PGF_{2\alpha}$) did, however, increase sharply between 125 and 145 days gestation indicating an increase in PGHS synthetic capacity towards late gestation. This study indicated an important role for PGHS rather than PLA_2 as the rate limiting enzyme of the synthetic capacity of ovine cotyledonary tissue to produce PGs. This observation led to increased focus on PGHS regulation and expression in late gestation intrauterine tissues in studies relating to parturition. PGHS is the rate-limiting step and, therefore, primarily determines the production of PGs within a tissue. An overview of the literature pertaining to the role of PGHS in fetal membranes and placenta will be outlined in a later section.

PGHS exists as two isoforms: PGHS-1 and PGHS-2. PGHS-1 is considered the constitutive isoform and is responsible for regulating vascular homeostasis, stomach function and renal water and sodium resorption (91). PGHS-1 can be detected in most tissues (8), is expressed in cultured cells at constant levels throughout the cell cycle (92). It has also been shown to be developmentally regulated (93) and it can be downregulated in endothelial cells in response to acidic fibroblast growth factor (94). PGHS-2 on the other hand, is not detectable in most mammalian tissue but its expression can be rapidly induced by cytokines, tumor promoters, mitogens and endotoxins. Cells which exhibit this rapid induction of PGHS-2 include fibroblasts (92, 95, 96), endothelial cells (97), monocytes (98) and ovarian follicles (99).

PGHS–1 and PGHS–2 are encoded by separate genes located on separate chromosomes (100). PGHS-1 and PGHS-2 share 60% sequence homology within a species. All amino acids identified as important for catalysis are conserved in both isoforms and the kinetic properties of the reactions catalyzed by the two are similar (91). The key differences

between the two isozymes are a cassette of 18 amino acids near the carboxy-terminus in PGHS-2 that is absent in PGHS-1, and a cassette of 17 amino acids near the amino-terminus in PGHS-1 that is absent in PGHS-2 (91). Both PGHS-1 and PGHS–2 are integral membrane proteins with three amphipathic α-helices in the amino-terminus that act as anchors in the membrane (101). However, the intracellular locations of these enzymes may be different. PGHS-1 has been located on the lumenal side of the endoplasmic reticulum (ER) (102, 103) while PGHS-2 is located on both the ER and the nuclear membrane (102). The location of PGHS-2 on the nuclear envelope may indicate a role in transcriptional regulation and an ability to affect cell replication and differentiation (8).

PGHS-1 and PGHS-2 most efficiently metabolize the 20-carbon arachidonate (20:4 ω6) and dihomo-γ-linoleate (20:3 ω6). Both PGHS isoforms catalyze two separate reactions in the conversion of AA to the PG precursor, PGH_2. The first reaction, the cyclooxygenase reaction, converts AA to PGG_2 by means of the addition of two oxygen molecules. The second reaction, in which PGG_2 undergoes a $2e^-$ reduction to PGH_2, is called the peroxidase reaction (104, 105). These reactions occur at two separate substrate binding sites and are important sites of PG synthesis inhibition. Both PGHS isoforms undergo suicide inactivation. It has been reported that, on average, each cyclooxygenase molecule consumes approximately 1400 arachidonate molecules before becoming suicide inactivated (106). It has been proposed that the mechanism of suicide inactivation may involve destructive side reactions of enzyme intermediates in the cyclooxygenase catalytic cycle (107).

1.5.2.1 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit PGHS activity by competing with AA for the cyclooxygenase reaction binding site (108, 109). These drugs can be isoform specific or non-specific (110). In 1974, Zuckerman was the first to report the effectiveness of NSAIDs (indomethacin specifically) in delaying human preterm labor (111). However, NSAIDs readily cross the placenta (112), inhibit fetal PG synthesis and disrupt fetal physiological processes (discussed below). Isoform specific inhibition may eliminate effects on fetal health while maintaining pregnancy.

1.5.3 Factors Regulating PGHS

As described earlier, in addition to their variation at the carboxy- and amino- termini, PGHS–1 functions primarily to regulate homeostasis while the PGHS-2 gene is inducible. PGHS-1 can be upregulated 3-4 fold whereas PGHS-2 can be upregulated 80 fold (113, 114). Studies have indicated that both the PGHS-1 and PGHS-2 genes contain regulatory response elements. PGHS-1 contains response elements for NF-IL6 and shear stress while PGHS-2 contains cyclic adenosine mono-phosphate (cAMP), interleukin-6 (IL-6), nuclear factor-κB (NF-κB, a transcription factor) and glucocorticoids (115).

1.5.4 Prostaglandin E_2 and $F_{2\alpha}$-Synthases (PGES/PGFS)

Prostaglandin E_2-synthase and prostaglandin $F_{2\alpha}$-synthase (PGES/PGFS) convert the endoperoxide intermediate PGH_2 into PGE_2 and $PGF_{2\alpha}$, respectively. Little is known about their regulation and their roles in regulating PG synthesis. Unezaki *et al.* (1996) determined that PGF synthase levels in the placenta were much lower than in the uterus at term (116). Additional information regarding the expression, localization, and change in activity of these enzymes with term or preterm labor is lacking.

1.5.5 Prostaglandin Metabolism

NAD^+-dependent prostaglandin dehydrogenase (PGDH) catalyzes the reversible oxidation of the 15-hydroxy groups of both PGE_2 and $PGF_{2\alpha}$ thereby regulating their bioavailability (117-119). The resulting 15-keto derivatives are inactive (12). In relation to intrauterine metabolism, PGDH has been localized to human placental trophoblast and syncytiotrophoblast (120) and PGDH activity is high in this tissue. A decrease in PGDH activity in the placenta in late gestation (121) may contribute to the increase in circulating PGs seen at this time (122-124). However, Sangha *et al.* (1994) showed that placental PGDH mRNA expression did not change at term or at preterm labor, with or without infection (125).

Amnion PGHS-2 mRNA expression, PGHS activity and PG production have been shown to increase in late gestation and with labor (126-131). However, chorionic PG metabolism may interfere with the transfer of amniotic PGs and limit their access to the putative target organ: the myometrium. Indeed, PGDH specific activity is high in chorion and decidual tissue obtained before and after labor, whereas, amnion PGDH

activity remains low (89). As well, although amnion and decidual PG synthesis *in vivo* increased in tissues collected at spontaneous labor this increase is accompanied by a high rate of PG metabolism in the chorion (132). Additionally, irPGDH has not been localized to amnion or decidua stromal cells, but PGDH is detectable in the chorionic trophoblast (120).

Recent evidence indicates that chorion PGDH activity decreases in the chorion adjacent to the lower uterine segment (133). In addition, PGFM production and PGDH mRNA expression may be reduced significantly in chorionic cultured cells and explants at the time of term labor (134). In some cases of idiopathic preterm labor there is a reduction in irPGDH staining, PGDH activity and mRNA expression in chorionic trophoblasts (125). As well, in cases of preterm labor with an underlying infection, there is a reduction in irPGDH, PGDH mRNA expression and PGDH-specific activity (135). This is thought to be due to the loss of chorionic trophoblast cells associated with infection induced preterm labor (133).

There is conflicting evidence surrounding PGE_2 transfer across full-thickness membranes *in vitro*, and how this changes with labor. PGE_2 produced by the amnion has been shown to cross the chorion in tissues collected after term labor (136). However, other studies suggest that the increase in amnion PGs at labor does not result in an increase in PG concentrations on the maternal side of full thickness membranes (137) and PGE_2 may not remain intact during transfer across full thickness membranes (138).

1.5.6 Prostaglandin Receptors

PGs exert their biological effects through seven transmembrane, G-protein-coupled receptors (139, 140). Typically, these act to change intracellular Ca^{2+} concentrations. The biological activity of $PGF_{2\alpha}$ is mediated through the FP receptor which acts to elevate intracellular Ca^{2+} concentrations (141) leading to increased muscle contractility. PGE_2 can act through 4 receptor subtypes: $EP_{1\text{-}4}$. Ligand interaction with EP_1 and EP_3 causes a contractile response while interaction with EP_2 and EP_4 induces relaxation. The location, abundance and regulation of these receptors may affect changes in uterine contractility and susceptibility to agonists with increasing gestational age and labor (142). Recently, Sugimoto *et al.* (1997) reported that FP receptor knockout mice failed to

deliver at term and did not show the decline in serum P_4 levels that precedes parturition in this species (48). Ovariectomy on gestational day 19 allowed successful delivery in these FP knockouts. This study confirmed that parturition is initiated when $PGF_{2\alpha}$ interacts with ovarian FP to induce luteolysis. In the mouse, evidence suggests that uterine EP receptors at term labor are of the EP_1 and EP_3 subtypes (143) and it has been shown that the concentration of FP and EP are highest in the fundus, or central region of the uterus, and lowest or non-detectable in the cervical region (144) in the rat. The location and gestational changes of either FP or EP receptors in fetal membranes and placenta are not known.

1.5.7 Steroid Interactions with the Prostaglandin Synthetic Pathway

Progesterone may control PG levels by influencing substrate availability (PLA_2), substrate conversion into biologically active metabolites (PGHS) or by upregulating PG metabolism (PGDH). Wu *et al.* (1997) treated ovariectomized ewes with E_2, P_4 or a combination of the two to determine the effect of these steroids on PGHS-1 and PGHS–2 mRNA and protein levels in the myometrium and endometrium (145). The results of this study indicated that in the ovine myometrium, PGHS-2 mRNA and protein levels were upregulated by E_2. However, the E_2 + P_4 treatment induced an upregulation of only PGHS-2 protein levels and P_4 treatment alone had no effect on either PGHS-2 mRNA or protein levels. Neither PGHS-1 mRNA nor protein levels changed with either treatment in the myometrium or the endometrium. In the endometrium, all treatments induced increased PGHS-2 protein levels whereas PGHS-2 mRNA expression was not detectable in this tissue. This study indicated that E_2 and P_4 effects on uterine PGHS mRNA and protein levels can vary with location. Additionally, P_4 has been shown to suppress PGHS-2 mRNA expression in bovine endometrial cells (146, 147), myometrial myocytes (146) cervix (148), in human endometrium (149) and in rat preovulatory follicles (150). P_4 may also suppress mouse uterine PGHS-2 (151). These studies suggest that P_4 inhibits PGHS-2 although, the effect of P_4 or E_2 on either fetal membrane or placental PGHS mRNA expression has not been investigated.

As mentioned previously, the chorion displays high PGDH activity and presents a metabolic barrier to amnion PGs. PGDH activity has been shown to be regulated by the opposing actions of P_4 and cortisol (89). Cultured decidual explants from women who

had received RU486, a P_4 receptor antagonist, *in vivo* had a higher PGE/PGEM ratio than control tissue (152). Additionally, a sharp reduction in PGDH activity was reported in decidual tissue collected from women after RU 486 administration in early pregnancy (153). In a study by Patel *et al.* (1999) cortisol inhibited PGDH activity and mRNA expression in placental and chorionic trophoblast (154). In the same study, P_4 agonists increased PGDH activity while P_4 antagonists had an inhibitory effect on PGDH activity and mRNA expression. An additional study by Cheng *et al.* (1993) measured PGE_2 and PGEM concentrations in chorionic villi and decidual tissue after administration of RU486 to women in the first trimester of pregnancy (155). They observed that RU486 had little effect on PGE_2 or PGEM concentrations or tissue distribution in the chorion. In the decidua, PGE_2 levels increased after RU486 treatment. The most prominent change was observed in small decidual blood vessels where the PGE/PGEM ratio increased with RU486 treatment. These experiments indicate that a key role of P_4 in pregnancy may be to maintain low PG levels by upregulating PGDH mRNA expression and activity. Overall, the studies outline in this section indicate that steroids are involved in regulating the PG biosynthetic pathway within the uterine environment.

1.6 Prostaglandins and Labor

Previous sections have reviewed the importance of the enzymes involved in the synthesis and metabolism of intrauterine PGs. The level of PGs within a tissue is also an important consideration. PG concentrations in intrauterine tissue, plasma and amniotic fluid increase around the time of labor, myometrial tissue exposed to PGs demonstrates increased contractility and NSAID administration results in delayed labor onset (27, 156, 157). In humans, sheep and rhesus monkeys, amniotic fluid and maternal plasma PGE_2 and $PGF_{2\alpha}$ concentrations increased prior to (122-124), and with the onset of labor (157, 158), indicating a role for PGs in both the initiation and progression of labor. Human placenta, amnion and chorion synthesize PGE_2 while the decidua synthesize both PGE_2 and $PGF_{2\alpha}$ (74, 89, 159). Placental PGE_2 can be released into both the fetal and maternal circulation and has been shown to promote uterine contractility and cervical ripening and increase uterine blood flow (159) (possibly through two types of PGE receptors). Similarly, the sheep placenta primarily synthesizes PGE_2. Fetal sheep plasma PGE_2 levels increase in late gestation, play a role in the initiation of labor, and serve as

feedback signals to the fetal HPA-axis (160, 161). In murine fetal membranes and placenta, $PGF_{2\alpha}$ and PGE_2 have been detected (162). In addition, high levels of 6-keto-$PGF_{1\alpha}$ (a stable PGI_2 metabolite) have been recorded in these tissues (162). The role of PGI_2 in murine labor has not been determined although it may play a role in the relaxation of human myometrial cells (163).

1.7 Other Roles for Intrauterine PGs

1.7.1 Placental Prostaglandins

PGs produced by the placenta may play a role in aspects of fetal and intrauterine physiology, other than the control of labor. In an isolated perfused human placenta collected at term, PGE_2 was synthesized and released at higher concentrations into the fetal reservoir than the maternal reservoir (159). The opposite was observed for $PGF_{2\alpha}$. Overall, the amount of PGE_2 released by the human placenta was greater than the amount of $PGF_{2\alpha}$. The PGE_2 released into the fetal circulation increased the fetal perfusion pressure by up to 200% indicating that placental PGE_2 is important in the constriction of placental vasculature. Additional studies showed that PGE_2 dilated uterine blood vessels (164), but had a vasoconstrictor effect on the fetal side of the placenta (165). This apparent dual role for placental PGE_2 may be due to variation in EP receptor subtypes within the intrauterine tissues. These authors did not address the role of placental $PGF_{2\alpha}$ which, despite lower recorded concentrations, may act in coordination with increased FP activity, thus enabling it to exert increased biological activity. Additional studies indicated that in the human placenta, PGE_2 production was higher than $PGF_{2\alpha}$ and that PGE_2 metabolism was low (166, 167).

In contrast to the studies outlined above, PGE_2 and $PGF_{2\alpha}$ have been shown to be produced by the human placenta in roughly equal amounts while production by the maternal side was four times greater than that of the fetal side (168). Elder *et al.* (1985) proposed that vasodilatory PGE_2 produced by the placenta may offset the vasoconstrictive effect of angiotensin (169). In that study, angiotensin infusion had no hypertensive effect on the placental vasculature of normal pregnant women. However, the subsequent administration of a PGHS inhibitor removed the vasodilatory effect of PGE_2 and caused hypertension.

1.7.2 Prostaglandins and Fetal Physiology

Maternal NSAID administration for the purposes of delaying labor onset has given further insight into the importance of PGs in fetal organ function and development, since, as mentioned previously, NSAIDs readily cross the placenta and enter fetal circulation (112). This has been shown to cause oligohydramnios (low amniotic fluid volume due to suppressed kidney function) (170), increased neonatal risk of necrotizing enterocolitis (necrosis of the gut) (171) and premature closure of the ductus arteroisus (172). Additionally, both PGE_2 and PGI_2 are regulators of perinatal thermogenesis (173). As well PGE_2 is an important regulator of fetal breathing movements and plays a key role in HPA-feedback regulation in the sheep (160, 161). Recent evidence from our laboratory indicates that PGHS-2 mRNA expression is upregulated in a number of human fetal organs in late gestation and with term labor (174). In addition, murine fetal kidneys show increased PGHS-2 protein content with increasing nephrogenesis (175). It has been well substantiated that PGs are important in the fetus. However, it is unclear whether PGs that are important for fetal physiology originate within the organs and act locally to modulate factors involved in cellular differentiation and function or whether they originate from distal sources such as the placenta. A recent study by Reese *et al.* (2000) suggested that PGs important for ductus arteriosus patency originate from distal (*i.e.* placenta), rather than local sources (162).

1.8 Fetal Membrane and Placental PGHS

Since PGHS is the rate limiting enzymatic step in the synthesis of PGs and isoform specific inhibition may lower the rate of preterm birth and improve newborn health, investigations into the relative roles of PGHS-1 and PGHS–2 in the production of intrauterine PGs are important. The following section will outline results from studies performed on the human, sheep and mouse.

1.8.1 Human PGHS

A number of studies have indicated that fetal membranes are a key source for intrauterine PGs in humans during late gestation and term labor. Human amnion PGHS specific activity increases in late gestation prior to labor onset and in association with term labor

(176, 177). In addition, amnion PGHS-2 mRNA (131) and protein levels increase significantly with gestation and double with labor (126-130). The rise in human amnion PGHS-2 mRNA and protein levels positively correlates with increased PGHS specific activity (127, 177) and PGE_2 levels (130). These findings support the idea that PGHS-2 is important in human amnion PG production. Furthermore, PGHS-2-specific inhibition effectively inhibits PGE_2 formation in human amnion, while PGHS-1-specific inhibition has no effect on PGE_2 levels (130). PGHS-1 mRNA expression is detectable in the amnion, however, in contrast to PGHS-2, it does not increase at term or correlate with PGHS activity or tissue PG levels (127). Interestingly, both PGHS-1 and PGHS-2 increases after preterm labor in human amnion and both contribute to the increase in PGHS activity seen at preterm labor (131).

In human chorion, both PGHS-1 (124) and PGHS-2 mRNA (124, 178) and protein levels (178) increased in late gestation, prior to (178) and with term labor (179). However, only PGHS-2 mRNA expression correlates with increased PGHS activity (124, 179). Slater *et al.* (1999) found no increase in either PGHS-1 mRNA or protein levels (178). Changes in PGHS mRNA expression at term have been localized primarily to epithelial and mesenchymal cells (124). These data suggest a key role for chorionic PGHS-2, not PGHS-1, in the production of PGs in late gestation and at term labor. Human preterm labor has been associated with increased PGHS-1 and PGHS-2 mRNA levels, as well as PGHS-specific activity (124) in the chorion. However, unlike term labor, these changes have been localized only to the fibroblast cells of the chorionic mesenchyme (180).

In human placental tissue, PGHS (non-isoform selective) has been localized to the apical border of the villous syncytiotrophoblast and extra-villous trophoblast in term and preterm placentas irrespective of labor (181). PGHS-1 mRNA and protein levels are not detectable at either cesarean section or with spontaneous labor at term (182). In contrast, PGHS-2 mRNA and protein levels have been detected in human placental tissue, but, similar to PGHS-1, levels do not differ between the cesarean section and spontaneous labor groups (182). These results suggest that PGHS-2 is the dominant isoform in the human placenta, but its role at term and preterm labor is not clear.

As mentioned previously, the decidua is intimately associated with the chorion and the myometrium and serves as an important interface for fetal-maternal hormonal

communication (25). Although some studies have shown that PGE_2 and $PGF_{2\alpha}$ production increases in human decidua with term labor (183, 184), neither PGHS-1 nor PGHS-2 mRNA expression (185) or PGHS-specific activity (131, 185) increase in the human decidua at term. This may indicate the influence of other enzymatic steps in the AA cascade, the PG synthase enzymes, in the control of decidual PG production.

Studies in non-human primates indicate that PGHS-2 mRNA expression increases the decidua before labor and in the amnion during labor. In addition, no increase in chorion or placental PGHS-2 mRNA had been observed (186). The regulation and origin of PGs for labor may differ among primate species.

1.8.2 Ovine PGHS

In the sheep, the cotyledon appears to be the main source of intrauterine PGs. In 1988, Rice and Thorburn determined that the ability of cotyledonary microsomes to metabolize AA increased 25 fold between 100-145 days (gestational length=145 days) gestation and that the main products were PGE_2 and $PGF_{2\alpha}$ (187). This indicated that PGHS activity increased in cotyledonary tissue in late gestation.

In the sheep model, glucocorticoids are used to precisely control the timing of labor in order to determine the expression of the factors involved in labor initiation. Glucocorticoids (dexamethasone or betamethasone) administered on gestational day (GD) 131, results in labor within 57 hours (188). A number of reports indicate that PGHS activity and PGHS-2 mRNA and protein levels increase in cotyledonary tissue in late gestation, with term labor and with glucocorticoid-induced preterm labor (188-190). In addition, PGHS-2 mRNA and protein expression have been localized to ovine trophoblast cells (191). Conversely, PGHS-1 mRNA and protein expression are measurable in the cotyledons but neither increases with term or glucocorticoid-induced labor (192-194).

In the amnion and chorioallantois of the sheep, PGHS activity is measurable and correlates with PGHS-1 mRNA and protein levels. However, neither PGHS activity nor PGHS-1 mRNA or protein levels increased with term labor or glucocorticoid-induced preterm labor (190). In contrast to PGHS-1, PGHS-2 mRNA expression is not detectable in the amnion or chorioallantois and is not induced with labor (190, 194).

The findings summarized above indicate that the rise in ovine placental PGs is due to PGHS-2 induction in coordination with the mechanisms involved in labor (as indicated by glucocorticoid-induced labor). In addition, a recent study by Poore *et al.* (1999) reported that PGHS-2 specific inhibition by nimesulide delayed glucocorticoid-induced delivery in sheep by 17 hours and the increase in fetal and maternal PG concentrations usually seen at this time was completely abolished (195). Interestingly, in both humans and sheep, the synthetic capability of the fetal membranes and placenta seem to be important for labor initiation.

Recent studies by Gyomorey *et al.* (2000) and Wu *et al.* (1999) suggested that the expression of PGHS-2 mRNA and protein may occur in a temporal pattern from fetal to maternal sheep intrauterine tissues (191, 196). Both studies showed that PGHS-2 mRNA and protein levels increased gradually in the sheep placenta from 115 days gestation and the same pattern is seen slightly later in the endometrium from 131 days gestation. In contrast, myometrial PGHS-2 mRNA and protein levels increased only during labor. Both studies demonstrated a fetal to maternal progression of PGHS-2 in late gestation and with labor and provide evidence for fetal control of PGHS expression in fetal and maternal intrauterine tissues.

1.8.3 Murine PGHS

Recent knockout studies have investigated the relative roles of PGHS-1 and PGHS-2 in murine labor. As discussed earlier, FP knockout mice fail to enter labor at term (48). Recently, Tsuboi and colleagues (2000) determined that in FP knockout mice, uterine PGHS-1 mRNA expression increases between GD 15 and GD 17 coinciding with the normal timing of the luteolytic process (151). However, unlike wildtype mice in which uterine PGHS-2 mRNA expression increased with term labor, PGHS-2 mRNA expression does not appear to increase with term labor in the FP knockout dams. Therefore, an early rise in PGHS-1 appears to be involved in precipitating luteolysis which leads to a later rise in the PGHS-2 isoform which is important for uterine contractility.

In PGHS-2 knockouts ovulation and blastocyst implantation are impaired (197, 198). Therefore, details on the involvement of PGHS-2 in murine parturition are impossible to

determine using knockout models. Administration of the PGHS-2 specific inhibitor, however, (celecoxib) is effective in delaying the onset of labor (dams delivered on day 20) (162). Labor in PGHS-1 knockouts is also delayed: dams delivered on day 22 and fetal health is poor with few to none surviving over 24 hours (162). Exogenous administration of $PGF_{2\alpha}$ on day 18.5-19.0 resulted in normal labor and viable fetuses. Thus, PGHS-1 knockouts lack the $PGF_{2\alpha}$ necessary for luteolysis. As well, poor fetal health in the knockout litters is caused by the lengthened gestation, not by PGHS-1 involvement in developmental processes. Blastocyst implantation also provides information about fetal and maternal roles in labor onset in the murine model. PGHS-1 knockout dams implanted with PGHS-1 wildtype blastocysts deliver on day 19 (199). In contrast, PGHS-1 wildtype dams implanted with PGHS-1 knockout blastocysts also deliver on day 19 (162). These data indicate that both PGHS-1 and PGHS-2 are necessary for labor at term in the mouse and that fetal PGHS-1 is sufficient but not necessary for labor onset at term.

1.9 Experimental Rationale: The Mouse As a Model for Parturition

1.9.1 Mouse Pre-term Labor

In the mouse, labor does not occur naturally before term. Therefore, in order to study preterm labor in a mouse model, it is necessary to induce labor with such agents as lipopolysaccaride (LPS) (114, 200) and RU486 (PR antagonist) (113) or by ovariectomy (201). An ethanol-induced model of preterm birth developed by Cook and Randall in 1997 was used in this study (202). Ethanol at a dose of 6 mg/kg administered on GD 16 results in labor on GD 17.5 ± 0.01 with an incidence of 93% (202). Fetal health at preterm birth is poor but can be regained after NSAID administration (202). Therefore, fetal health is diminished by early labor, not ethanol administration.

Progesterone is important in the control of murine labor (48, 86, 199, 203). The rationale for the use of agents to induce artificial early labor is validated if the factors known to be involved in labor are upregulated during the process of early labor and thereby model normal labor-related physiological conditions. Indeed, both $PGF_{2\alpha}$ and PGE_2 have been shown to increase in uterine tissue collected from dams in ethanol-induced preterm labor and this is in coordination with early P_4 withdrawal as well as upregulation of uterine

PGHS-2 mRNA levels (204). Furthermore, ethanol-induced preterm labor could be antagonized by pretreatment with either P_4 or nimesulide (PGHS-2-specific inhibitor) (204). In this model, the early P_4 withdrawal may be due to a potentiated luteolytic process rather than triggered luteolysis because the ethanol is administered after the onset of corpus luteum regression (202). Interestingly, other factors important for labor onset including OTR, FP and Cx-43 mRNA levels increased during ethanol-induced preterm labor (203). Whether the expression of these uterine activation factors occurs is upregulated or not provides information about the endocrine differences between term and preterm labor.

1.10 Summary and Objectives

Animal models are a valuable research tool that allow for manipulations that are unethical in the human. Since the mouse is the only model available for genetic manipulation studies, it is important to understand the endocrine interactions leading to labor onset in this species. Studies in humans and sheep indicate that fetal membrane and placental PGs are important for the initiation of labor. Further characterization of the mouse as a model for parturition is required because the dynamics of PG synthesis in murine fetal membranes and placenta are not known. Therefore, the **first objective** of this study is to describe the profile of PGHS-1 and PGHS–2 mRNA levels in late gestation and at term and ethanol-induced preterm labor in murine fetal membranes and placenta. It has been shown that P_4 can regulate PG synthesis and it is known that P_4 is a key regulator of murine parturition. However, it is not known whether P_4 regulates fetal membrane and placental PG synthesis in the mouse. Therefore, the **second objective** of this study is to describe the profile of fetal membrane and placental PGE_2 and $PGF_{2\alpha}$ levels in late gestation and at term labor and to determine the effect of maternal progesterone supplementation on these PG levels in late gestation and at term labor.

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CHAPTER 2. The Expression of PGHS-1 and –2 mRNA in Murine Fetal Membranes and Placenta in Late Gestation and with Term and Ethanol-Induced Preterm Labor.

2.1 Introduction

Despite intensive research, the mechanisms involved in preterm labor initiation have not been fully elucidated and the incidence of preterm birth has not decreased. This problem continues to be associated with high neonatal mortality and morbidity (1). It has been well established that prostaglandins (PGs) are important regulators of parturition. Inhibition of PG synthesis delays the onset of labor, prolongs the duration of labor and reduces myometrial contractility (2, 3). In addition, PG administration induces labor by eliciting contractile effects on the pregnant uterus (4, 5). PGs are synthesized from arachidonic acid through the action of prostaglandin H-synthase (PGHS). This enzyme exists as two isoforms: PGHS-1 and PGHS-2. While PGHS-1 is constitutive and PGHS-2 is inducible, both have the same catalytic activity (6). PGHS is an important point of regulation in the PG biosynthetic pathway because it is the rate-limiting step (7).

Fetal membrane and placental PG synthesis increases in late gestation and with the onset of labor in a number of species. PGHS-2 mRNA and protein levels in correlation with PGHS-specific activity have been shown to increase in human amnion prior to and with the onset of labor (8-15). In human chorion, both PGHS-1 (16) and PGHS-2 mRNA (16, 17) and protein levels (17) increased in late gestation, prior to (17) and with term labor (18). However, only PGHS-2 mRNA expression correlates with increased PGHS-specific activity (16, 18). Finally, in human placental tissue, only PGHS-2 mRNA and protein expression have been detected, but neither have been shown to increase with labor at term (19). In the sheep, cotyledonary PGHS-2 mRNA and protein levels increase with term labor (20). However, in the amnion and allantois only PGHS-1 mRNA and protein expression (21, 22) have been detected, but have not been shown to change with labor (22). Interestingly, two studies have shown that in the sheep, PGHS-2 mRNA and

protein levels increase initially in the placenta followed by increasing endometrial and myometrial levels with labor onset (23, 24). These authors suggest a temporal pattern of PG synthesis in sheep from fetal to maternal intrauterine tissues around the time of labor. The studies outlined above provide clear evidence of an important role for fetal intrauterine PG synthesis around the time of labor onset in humans and sheep. In the murine uterus, a previous study performed in our laboratory found that PGHS-2 mRNA levels increase with term labor (PGHS-1 mRNA levels were not measured) (25, 26). Others have also indicated an important role for both PGHS-1 and PGHS–2 in murine parturition at term (27-29). However, nothing is known about the late gestational or labor related changes in fetal membrane or placental PGHS mRNA expression in the mouse.

Human preterm labor has been associated with increased PGHS-1 and PGHS-2 mRNA levels as well PGHS-specific activity in both amnion (15) and chorion (16, 30). In human placental tissue, PGHS (non-isoform selective) has been localized to trophoblast in preterm placentas irrespective of labor (31). Evidence in the sheep indicates that PGHS activity and PGHS-2 mRNA and protein levels increase in cotyledonary tissue in late gestation with glucocorticoid-induced preterm labor (20, 22, 32). These studies indicate that in human and sheep preterm labor, PG synthetic capacity increases.

In the mouse, preterm labor can be induced by such agents as lipopolysaccharide (LPS) (33, 34) and RU486 (PR antagonist) (35) or by ovariectomy (26). In the present study, an ethanol-induced model of preterm birth developed by Cook and Randall in 1997 was used (36). Ethanol at a dose of 6 mg/kg administered on GD 16 results in labor on GD 17.5 ± 0.01 with an incidence of 93% (36). A previous investigation in our laboratory showed that murine uterine PGHS-2 mRNA expression increased at ethanol-induced preterm labor (25) and others have indicated a role for PGHS-2 in LPS-induced preterm labor (37). However, nothing is known about the expression of PGHS mRNA in mouse fetal membranes and placenta with preterm labor.

Overall, information from other species (sheep and human) indicates that fetal intrauterine PG synthesis is upregulated around the time of term and preterm labor. However, the data on fetal membrane and placental PG synthesis is lacking in the mouse. Since the mouse is an important model because of its exclusive use in gene deletion studies, a clear understanding of murine intrauterine PG synthesis is necessary. The

present study was designed to determine the profile of PGHS-1 and PGHS-2 mRNA expression in murine fetal membrane and placental tissue in late gestation and with term and ethanol-induced preterm labor.

2.2 Materials and Methods

2.2.1 Breeding

Nulliparous 10-12 week old female C57BL/6J mice (The Jackson Laboratories, Bar Harbor, ME) were bred for these experiments. These animals were housed in a temperature and humidity-controlled room, with a daily 12 h light, 12 h dark cycle (lights on at 6:00 am). One to four females were placed in each cage containing one male for breeding, daily between 9:00 am and 11:00 am. After this time, females were examined for the presence of a seminal plug. Successfully bred females were weighed, housed individually and then weighed daily after GD 10 in order to monitor the dams for pregnancy progression. The day on which the seminal plug was present was considered day 0 of gestation.

2.2.2 Treatment

Pregnant dams were assigned to the following treatment groups: (1) untreated control (n=6/timepoint) or (2) ethanol treated (n=6/timepoint). Previous data indicated that dams treated with isocaloric sucrose as a vehicle for alcohol did not differ in uterine PG levels when compared to untreated dams, (36), therefore, untreated dams were used as the control group in this study. A dose of 6 mg/kg of ethanol was administered to pregnant dams intragastrically (i.g.) on GD 16 as previously described (36). Both term and preterm labor were defined as delivery of the first pup.

2.2.3 Tissue Collection

Fetal membranes and placentas were collected from pregnant mice at 9:00 am on GD 14, 16, 17.5 and after the delivery of the first pup at labor and ethanol-induced preterm labor. The normal length of gestation in this mouse strain is 19.3 ± 0.01 days (25) and ethanol-induced preterm delivery occurs on GD 17.5 ± 0.01 (36). The GD 17.5 untreated timepoint (not in labor) was used as the gestational age-matched control group for the ethanol-treated dams in preterm labor. Pregnant dams were killed by cervical dislocation. The abdomen was opened and the uterus was carefully dissected and washed in a

physiological (0.9%) saline solution. The uterus was then cut open taking care not to break the membrane sacs. Each placenta was gently pulled away from the uterine wall, the sac was broken and the membranes and placenta were removed from the fetus. The umbilical cord was cut close to the placenta to ensure that only placenta tissue was collected. The membranes were then removed at the interface between the fetal membranes and placenta. The tissues were snap frozen in liquid nitrogen and stored at -70°C until assayed.

2.2.4 RNA Extraction

Total RNA was extracted with Trizol RNA reagent (Gibco BRL, Burlington, ON). Frozen fetal membrane and placental tissue was ground to a fine powder in liquid nitrogen. Trizol was added to 0.05 g of ground sample and the tissue was homogenized on ice. Chloroform (200 µl) was added to each homogenate and the samples were centrifuged at 15,000 *x g* for 15 min. The supernatant was removed and added to an equal volume of isopropanol. The sample was vortexed, frozen on dry ice for 10 min, and centrifuged at 15,000 *x g* for 10 min. The supernatant was removed, the RNA pellet was washed with 1 ml of 75% ethanol and the sample was centrifuged at 15,000 *x g* for 5 min. Following centrifugation, the supernatant was removed and the pellet was allowed to air-dry for 5 min. Finally, the RNA pellet was dissolved in TE buffer (10mM Tris-Cl, pH=8.0 + 1mM EDTA, pH=8.0) and total RNA was quantified by spectrophotometric analysis at 260 nm. The integrity of the RNA was also confirmed by agarose gel-electrophoresis.

2.2.5 Generation of RNA probes

Levels of the constitutively expressed cyclophilin mRNA were measured to verify uniform abundance of cyclophilin in the different samples and across gestation. PGHS values were normalized to cyclophilin. Linearized pGT-PGHS-2, pGT-PGHS-1 and pTRI-Cyclophilin-Mouse plasmids were transcribed to make murine PGHS-2, PGHS-1 and cyclophilin antisense RNA probes. The pTRI-Cyclophilin-Mouse construct used for making the cyclophilin probe was obtained from Ambion. RNA probes were made as previously described (25). Briefly, 1 µg of linearized plasmid was transcribed with either T7, T3, or SP6 RNA polymerase under the following conditions: 5 µCi / µl α-^{32}P-CTP; 0.5 mM each of rATP, rTTP, and rGTP; 10 mM DTT; 40 mM Tris (pH 7.9); 6 mM

$MgCl_2$; 2 mM spermidine; 10 mM NaCl;, 1.2 U / µl RNasin Ribonuclease inhibitor; 0.1 µg / µl linearized DNA template; and 2 U / µl T7, T3 or SP6 polymerase. Following transcription, the DNA template was digested by adding 24 U RNasin Ribonuclease inhibitor, and 60 U DNase I. Following phenol/chloroform extraction, the RNA was precipitated with 100% ethanol using yeast tRNA as a carrier.

RNA pellets were resuspended in 20 µl formamide RNA buffer (80% formamide, 1 mM EDTA, pH 8.0, 0.1% bromophenol blue, 0.1% xylene cyanol), denatured by boiling for 5 min, placed on ice, and electrophoresed on a 6 % polyacrylamide, 8 M urea gel. Full-length transcripts (PGHS-2: 437 nt, PGHS-1: 320 nt and Cyclophilin: 165 nt) were cut out of the gel and eluted in 400 µl elution buffer (2 M ammonium acetate, 1 % SDS, and 25 µg / ml yeast tRNA) for 3 h at 37°C. RNA was then precipitated from the supernatant with 100 % ethanol. Following centrifugation at 14 000 $\times g$ for 10 min, the RNA pellet was resuspended in hybridization buffer (80 % formamide, 40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA). Two µl were counted in a liquid scintillation counter to determine incorporation.

2.2.6 RNase Protection Assay

Twenty µg of total murine placenta and fetal membrane RNA was hybridized to either 5 $\times 10^5$, 1 x 10^6 or 2 x 10^6 cpm of the appropriate probe in 30 µl hybridization buffer for 16 h at 55°C. Yeast tRNA was processed in the same manner as a negative control. Following hybridization, samples were digested with 2.3 µg / ml ribonuclease A and 300 U ribonuclease T1 in 300 µl ribonuclease digestion buffer (10 mM Tris-Cl, pH 7.5, 300 mM NaCl, 5 mM EDTA) for 30 min at 30°C. Ribonucleases were removed by treatment with 25 µg/ml proteinase K in the presence of 0.6 % SDS for 20 minutes at 37°C. Samples were phenol/chloroform extracted and precipitated with isopropanol using yeast tRNA as a carrier. Samples were centrifuged for 10 min at 14 000 $\times g$, resuspended in 8µl formamide RNA buffer and electrophoresed on a 6 % acrylamide, 8 M urea gel. The gel was dried, exposed to film for either 15h (PGHS) or 1h (cyclophilin - within the linear range) and analyzed by autoradiography. Any appearance of overexposure in the figures is an artifact of reproduction from original film. Protected bands were the following sizes: PGHS-2:362 nt, PGHS-1:231 nt and Cyclophilin:103 nt. Autoradiograms were analyzed by densitometry using the Biorad Fluor-S-Max multi-

imager. Background was subtracted for each lane. Densitometric measurements of PGHS mRNA were normalized to those of cyclophilin and values are expressed as percent cyclophilin. The normalized data have been expressed as normalized densitometric units (ndu) ± standard error of the mean (SEM).

2.2.7 Data Analysis

All normalized data were analyzed by one-way Analysis of Variance (ANOVA) with post-hoc analysis by Tukey's test. Statistically significant differences between the preterm labor group and its gestational age-matched control (GD 17.5) were determined using Student's t-test. Significance was achieved when $p<0.05$.

2.3 RESULTS

2.3.1 Fetal Membrane PGHS mRNA in Late Gestation and with Term Labor

Representative blots of fetal membrane PGHS mRNA and cyclophilin mRNA (term and preterm labor n=2/timepoint) are shown in Figures 2-1A and 2-2A. Normalized fetal membrane PGHS-1 mRNA expression (Figure 2-1B) tended to increase with advancing gestational age although the data were significantly different only between GD 14 (5 ± 3 ndu) and GD 19.3 (13 ± 2 ndu). Normalized fetal membrane PGHS-2 mRNA expression (Figure 2-2B) increased significantly between GD 17.5 (17 ± 3 ndu) and GD 19.3 (29 ± 4 ndu).

Normalized preterm fetal membrane PGHS-1 mRNA levels were compared to a gestational age-matched control (not in labor) on GD 17.5 (Figure 2-3B). Preterm PGHS-1 mRNA expression (22 ± 5 ndu) did not differ significantly from GD 17.5 values (11 ± 3 ndu). Similarly, preterm (15 ± 4 ndu) and GD 17.5 (17 ± 5 ndu) normalized PGHS-2 mRNA expression (Figure 2-3B) did not differ significantly.

2.3.2 Placental PGHS mRNA in Late Gestation and with Term Labor

Representative blots of placental PGHS and cyclophilin mRNA (term and preterm labor n=2/timepoint) are shown in Figures 2-4A and 2-5A. Normalized placental PGHS-1 mRNA expression (Figure 2-4B) did not change significantly with advancing gestational age or with term labor. In contrast, PGHS-2 mRNA expression (Figure 2-5B) increased

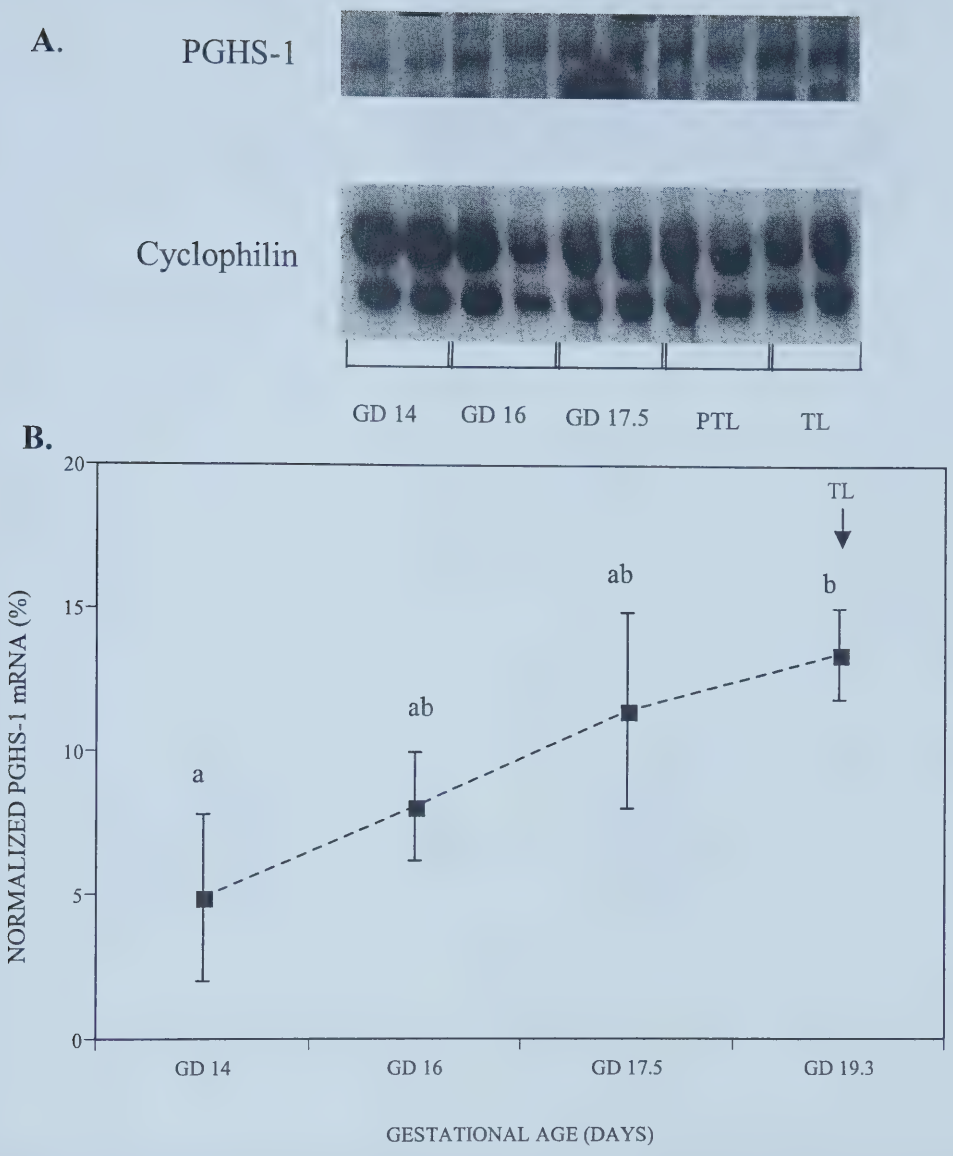

Figure 2-1 Murine fetal membrane PGHS-1 mRNA expression.

Fetal membrane PGHS-1 mRNA was measured by RPA and normalized to cyclophilin. A) Representative blot (n=2/timepoint) of PGHS-1 and cyclophilin mRNA, including term labor (TL) and preterm labor (PTL) groups. B) Normalized PGHS-1 mRNA (n=6/timepoint). Data were analyzed by one-way ANOVA with post-hoc analysis by Tukey's test ($p<0.05$). The only significant difference was observed between GD 14 and GD19.3 (term labor). Letters that differ indicate significance.

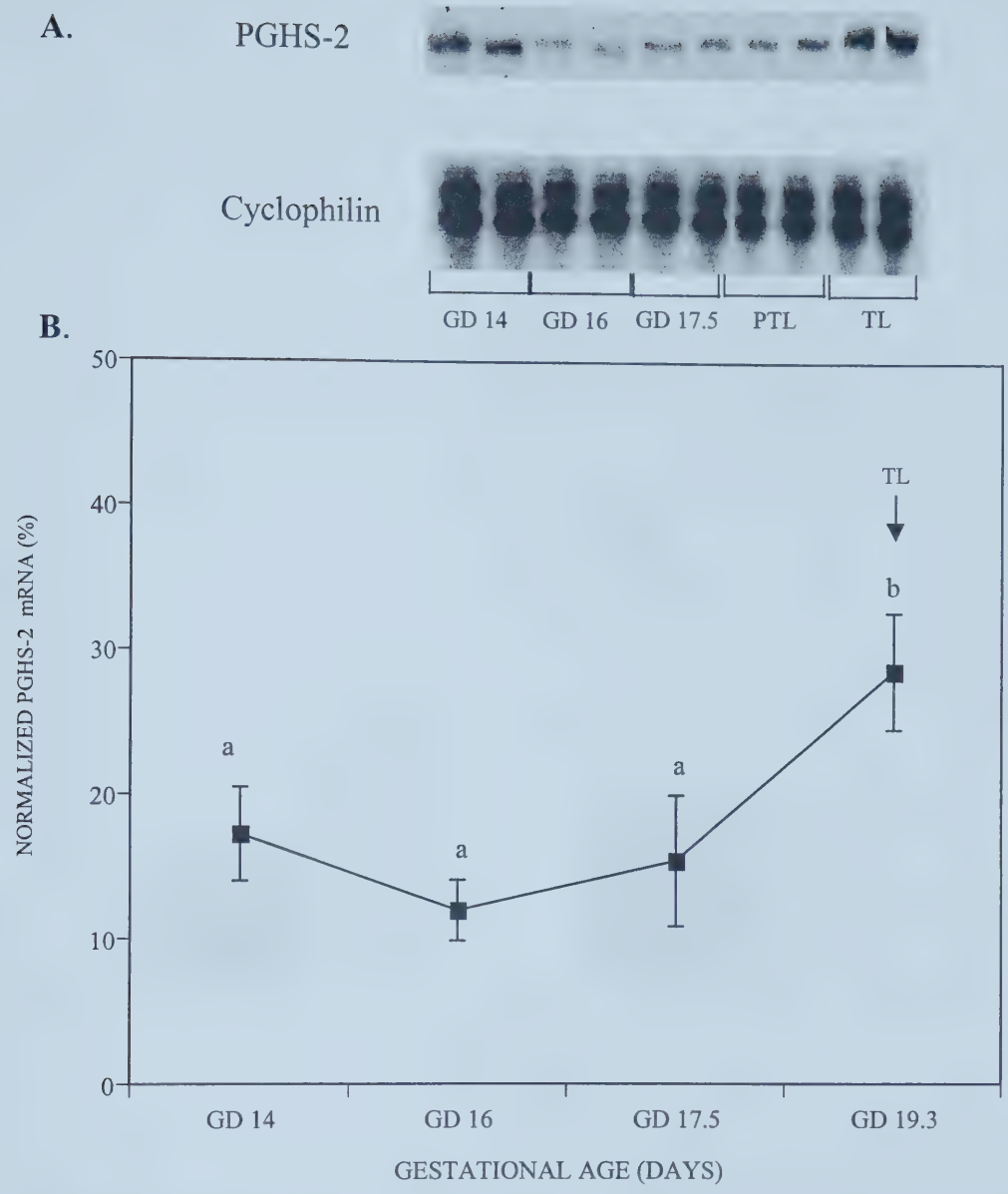

Figure 2-2 Murine fetal membrane PGHS-2 mRNA expression.

Fetal membrane PGHS-2 mRNA was measured by RPA and normalized to cyclophilin. A) Representative blot (n=2/timepoint) of PGHS-2 and cyclophilin mRNA including term labor (TL) and preterm labor (PTL) groups. B) Normalized PGHS-2 mRNA. Data were analyzed by one-way ANOVA with post-hoc analysis by Tukey's test ($p<0.05$). PGHS-2 mRNA increased significantly between GD 17.5 and GD19.3 (term labor). Letters that differ indicate significance.

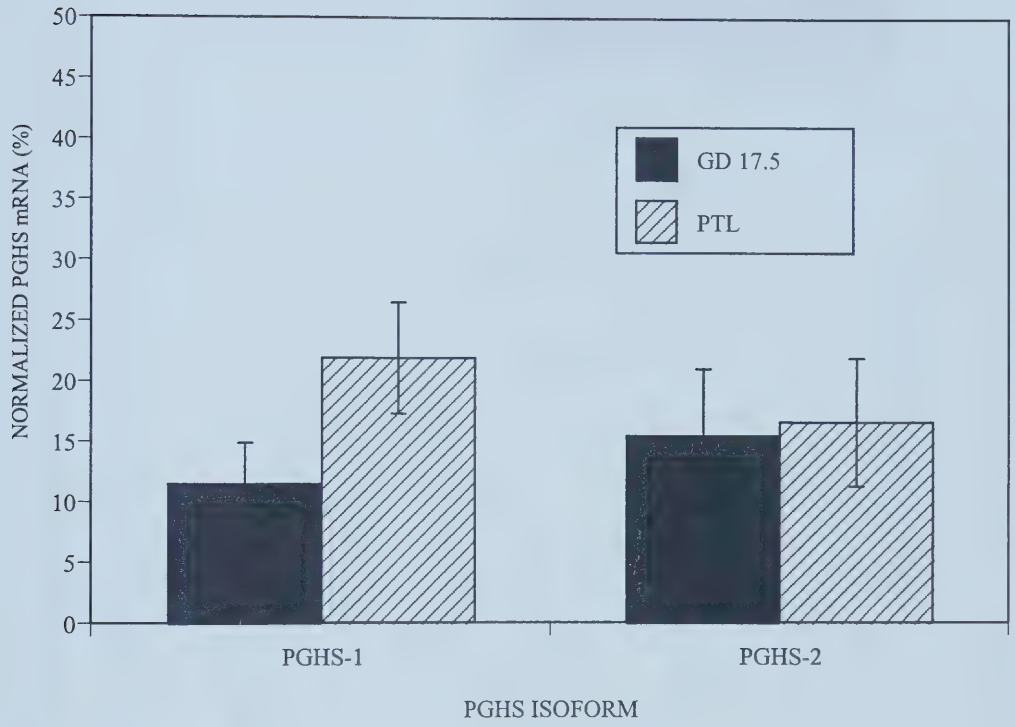

Figure 2-3 Preterm murine fetal membrane PGHS mRNA expression.

Fetal membrane PGHS-1 and PGHS-2 mRNA levels were measured by RPA and normalized to cyclophilin (n=6/timepoint). For the representative blots of these data (n=2/timepoint) refer to figure 2-1A and 2-2A. Data were analyzed by Student's t-test ($p<0.05$). There was no significant difference between PTL and it's gestational age-matched control (GD 17.5) for either PGHS-1 and PGHS-2 mRNA expression.

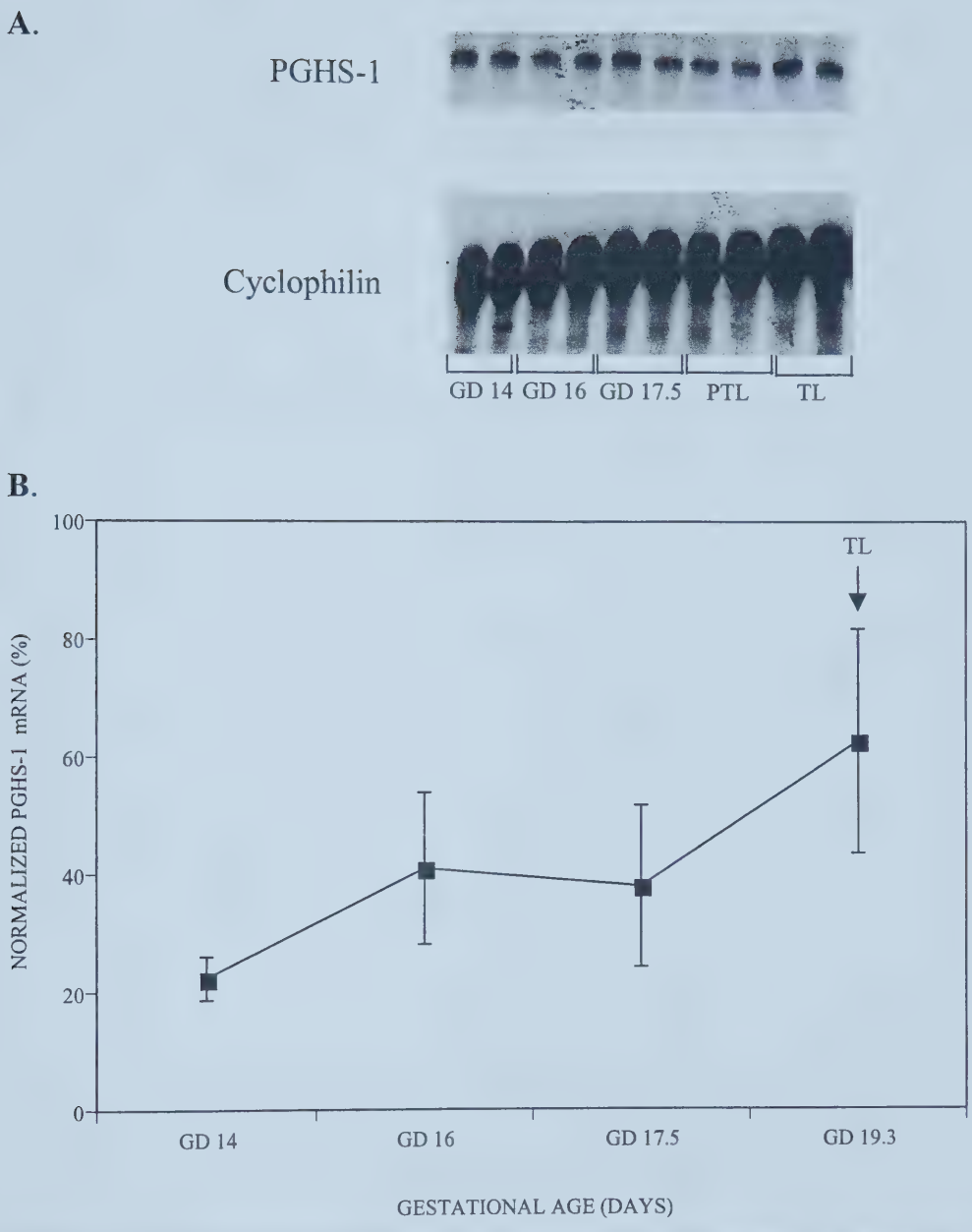

Figure 2-4 Murine placenta PGHS-1 mRNA expression.

Placental PGHS-1 mRNA was measured by RPA and normalized to cyclophilin. A) Representative blot (n=2/timepoint) of PGHS-1 and cyclophilin mRNA including term labor (TL) and preterm labor (PTL) groups. B) Normalized PGHS-2 mRNA (n=6/timepoint). Data were analyzed by one-way ANOVA ($p<0.05$). There was no significant change with increasing gestational age.

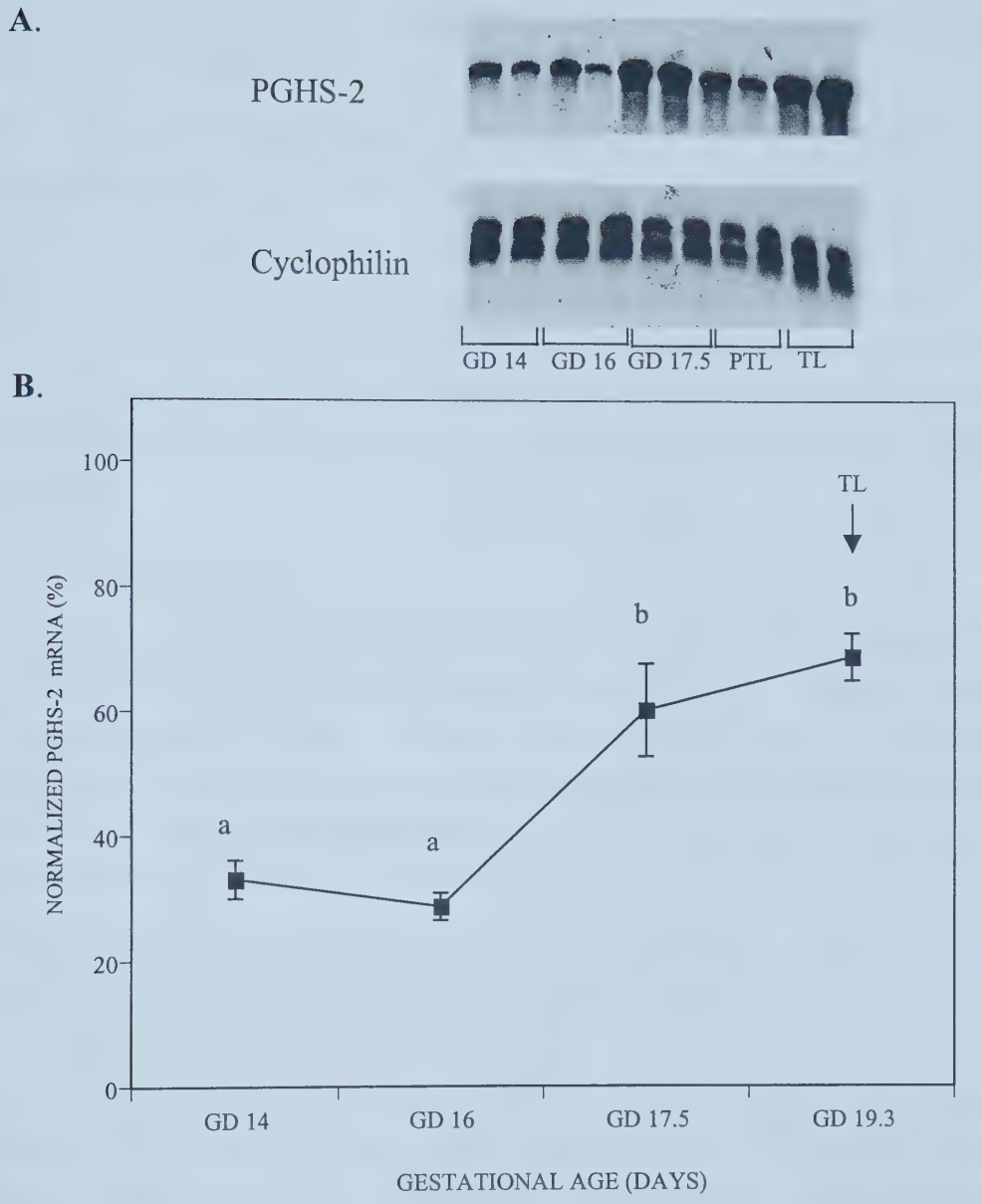

Figure 2-5 Murine placenta PGHS-2 mRNA expression.

Placental PGHS-2 mRNA was measured by RPA and normalized to cyclophilin. A) Representative blot (n=2) of PGHS-2 and cyclophilin mRNA including term labor (TL) and preterm labor (PTL) groups. B) Normalized PGHS-2 mRNA (n=6/timepoint). Data were analyzed by one-way ANOVA with post-hoc analysis by Tukey's test ($p<0.05$). A significant increase was observed between GD 16 and GD 17.5. No further increase was seen at GD 19.3 (TL). Letters that differ indicate significance.

significantly between GD 16 (29 ± 2 ndu) and GD 17.5 (60 ± 7 ndu). No further increase was observed at term labor (69 ± 4 ndu).

2.3.3 Placental PGHS mRNA with Preterm Labor

Normalized preterm placental PGHS-1 mRNA expression was compared to a gestational age-matched control (not in labor) on GD 17.5 (Figure 2-6). Preterm PGHS-1 mRNA levels (57 ± 20 ndu) did not differ significantly from GD 17.5 values (38 ± 14). Although the mean values of PGHS-1 mRNA levels were higher at preterm labor than its gestational age-matched control, this difference was not statistically significant. Preterm (56 ± 6 ndu) and GD 17.5 (60 ± 7 ndu) PGHS-2 mRNA levels (Figure 2-6) did not differ significantly.

2.4 DISCUSSION

In the present study, fetal membrane PGHS-1 and PGHS-2 mRNA expression and placental PGHS-2 mRNA expression increased in late gestation, indicating a possible role for late gestational increases in PGHS around the time of term labor. These results are consistent with data from human and sheep studies in which PGHS-1 and PGHS-2 mRNA and protein levels and PGHS-specific activity have been shown to increase in fetal membranes and placenta in late gestation (8-16, 18-20, 22, 30, 31, 38).

Previous studies have indicated an important role for both PGHS-1 and PGHS-2 in the onset of labor in the mouse. Uterine induction of PGHS-1 around GD 15 is considered to be important for the production of $PGF_{2\alpha}$ for the induction luteolysis while a later induction of uterine PGHS-2 is important for the production of PGs involved in uterine contractility (29). Studies involving PGHS-1 knockout dams found that these animals went into delayed labor on GD 22. Furthermore, the delayed labor can be offset by the administration of $PGF_{2\alpha}$ on day 18 (28). Another study reported that PGHS-2 specific inhibition delayed labor in wildtype dams as well (PGHS-2 knockout dams exhibit impaired ovulation and blastocyst implantation, therefore isoform specific inhibition must be used to study the role of PGHS-2 at term). These studies describe an important role for both PGHS-1 and PGHS-2 in the onset of murine labor. The present study is the first to describe the late gestational changes in murine fetal membrane and placental PGHS mRNA expression and indicate a possible important role for PGHS originating from

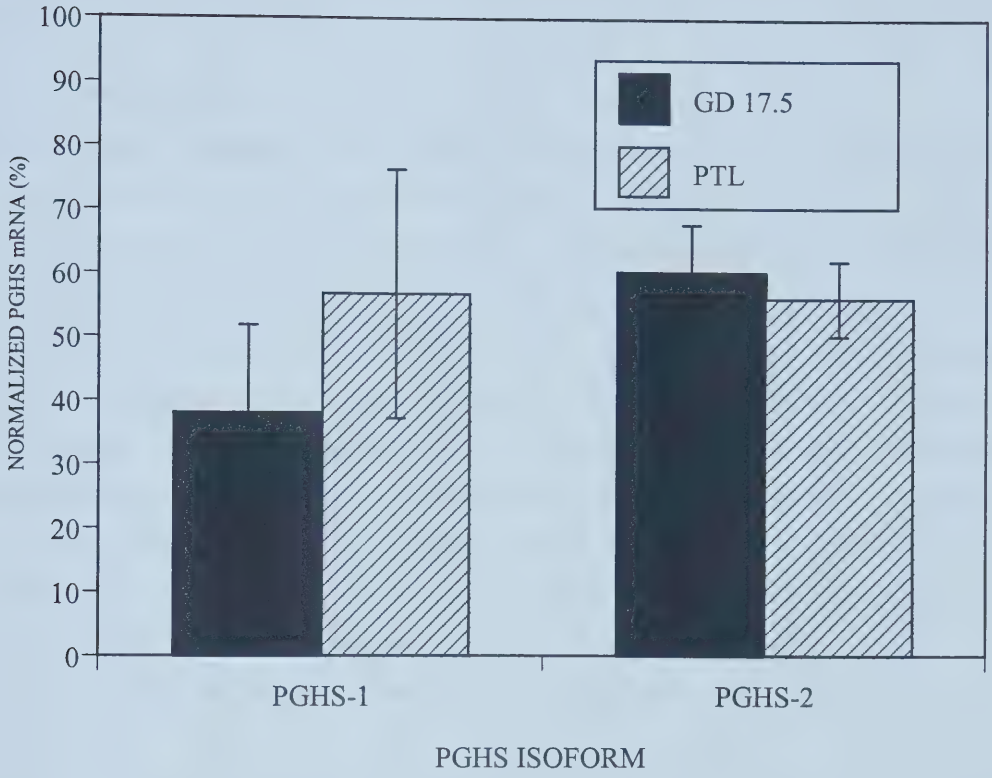

Figure 2-6 Preterm murine placenta PGHS mRNA expression.

Placental PGHS-1 and PGHS-2 mRNA levels were measured by RPA and normalized to cyclophilin (n=6/timepoint). For the representative blots of these data (n=2/timrpoint), refer to figure 2-4A and 2-5A. Data were analyzed by Student's t-test ($p<0.05$). There was no significant difference between PTL and it's gestational age-matched control (GD 17.5) for either PGHS-1 and PGHS-2 mRNA expression.

these tissues in this species. PGs originating from the fetal membranes and placenta may have local or endocrine effects including regulation of local vascular tone (placental and yolk sac vessels) (39-41), maintenance of ductus arteriosus patency (42) and induction of uterine contractility (4, 5, 43)

2.4.1 Fetal Membranes

In murine fetal membranes, PGHS-2 mRNA levels increased significantly between GD 17.5 and GD 19.3 while PGHS-1 mRNA increased gradually from GD 14, eventually reaching significance at GD 19.3 (term labor). These results indicate that both PGHS-1 and PGHS-2 may important in late gestation and at the time of term labor in murine fetal membranes. It is not unusual that PGHS-1 mRNA expression increased since, although, PGHS-2 is considered the inducible isoform, PGHS-1 can be upregulated as well (44, 45). Previous studies reported that PGHS-1 (28) and PGHS-2 (27) mRNA expression was abundant in murine decidua and that PGHS-2 mRNA was expressed in the yolk sac but neither PGHS-1 mRNA nor PGHS-2 mRNA expression was detectable in the amniotic membrane (27). Since the present study did not separate fetal membrane components, levels reported represent amnion, yolk sac and decidual levels. Other studies (described earlier) have shown that each membrane component may differentially express PGHS mRNA (10-13, 16-18, 20, 22, 30). As mentioned, uterine PGHS-1 induction at approximately GD 15 is considered important for the production of PGs for luteolysis. The induction of fetal membrane PGHS-1 mRNA observed in the present study at term labor may indicate differing roles for uterine and fetal membrane PGHS-1.

2.4.2 Placenta

In murine placenta, there was no significant increase in PGHS-1 mRNA levels with increasing gestational age. In contrast, PGHS-2 mRNA expression increased significantly between GD 16 and GD 17 and remained at GD 17 levels on GD 19.3. These results suggest that PGHS-2 is responsible for the production of PGs in late gestation and at term labor in murine placenta. Both PGHS-1 and PGHS-2 mRNA expression have been localized to the labyrinthine layer of the placenta at low levels during labor (27).

The changes observed in fetal membrane and placental PGHS mRNA expression around in late gestation, in the present study, may indicate a role for the fetally derived PGs in the onset of labor. Gross *et al.* (1998) reported that PGHS-1 was necessary in mice for labor at term since PGHS-1 knockout mice exhibited delayed labor (28). In order to determine the possibility of a role for the murine fetus he used blastocyst implantation techniques and determined that wildtype dams were able to deliver PGHS-1 knockout offspring at term. This indicated a need for maternal PGs only for the onset of labor. In contrast, Reese *et al.* (2000) showed that PGHS-1 knockout dams were able to successfully deliver wiltype and heterozygous fetuses at term (27). These studies indicated that fetal PGs are sufficient but not necessary for labor in the mouse. However, the role of fetal PGHS-2 was not considered. The results of the present study indicate that fetal membrane and placental PGHS-2 mRNA expression is upregulated around the time of term labor. Since these tissues are of fetal origin, this may indicate that fetal intrauterine PG synthesis is upregulated around the time of term labor and that the fetus contributes PGs to the intrauterine environment. However, since the tissue samples collected contained some maternal tissue (decidua and the maternal portion of the labyrinthine placenta), localization experiments need to be performed to determine whether the increase in PGHS-2 mRNA expression observed was a fetal effect.

2.4.3 Preterm PGHS

Preterm labor induced by ethanol administration on GD 16 precipitates an early decrease in plasma P_4 to levels observed at normal term labor (25). This key endocrine change may indicate that preterm labor represents early onset of term labor. Previous studies conducted in our laboratory determined that uterine PGHS-2 mRNA expression was upregulated at both term and ethanol-induced preterm labor. Furthermore, ethanol-induced preterm labor was effectively antagonized by pretreatment with nimesulide (PGHS-2 specific inhibition) (26). The present study indicated that there was no increase in either fetal membrane or placenta PGHS-1 or PGHS-2 mRNA expression at ethanol-induced preterm labor. Therefore, murine preterm and term labor may be initiated by different regulatory mechanisms and fetal membrane and placental PG synthesis may not have a role in the initiation of preterm labor. Gross *et al.* (2000) showed that uterine PGHS-2 mRNA expression was upregulated during LPS induced preterm labor in the

mouse and that PGHS-2 isoform specific inhibition attenuated preterm labor (37). Analysis by Western Immunoblot has shown that PGHS-2 protein levels increase in uterine tissue of endotoxin treated mice (46). In addition, in RU 486 induced preterm labor, uterine PGE_2 and $PGF_{2\alpha}$ production increased significantly (35) with the onset of preterm labor. These studies did not measure fetal membrane and placental PG synthesis and did not account for fetally derived PGs. Preterm labor in the human has been characterized by increased chorion and amniotic PGHS-1 and PGHS-2 mRNA expression and PGHS activity (16, 30). In addition, PGHS mRNA expression was localized to the mesenchymal tissue components at preterm labor, while at term labor PGHS mRNA expression increased in both mesenchymal and epithelial tissue components. In sheep glucocorticoid-induced preterm labor has been associated with increased PGHS activity and PGHS-2 mRNA and protein levels in cotyledonary tissue (20, 22, 32). In order to confirm the role of PGHS-1 and PGHS-2 in our model of murine ethanol-induced preterm labor, further investigation is needed to determine PGHS protein levels and PGHS-specific activity as well as PG levels.

A number of factors have been shown to regulate PGHS in intrauterine tissues. PGHS-1 is generally considered to be constitutive while PGHS-2 expression can be upregulated by cytokines, growth factors and tumor promoters (47). PGE_2 and $PGF_{2\alpha}$ production has been shown to increase in amnion and endometrial stromal cells in response to dexamethasone treatment (48), and glucocorticoids have been shown to upregulate PGHS-2 but not PGHS-1 mRNA expression in human amnion cells in culture (49). Evidence of P_4 suppression of PGHS-2 has been reported in bovine endometrial cells (50, 51), myometrial myocytes (50), in the cervix (52), in human endometrium (53) and in rat preovulatory follicles (54). P_4 may also suppress mouse uterine PGHS-2 (29).

2.4.4 Summary

The data from the present study, combined with information from earlier studies conducted in this laboratory indicate that preterm labor may be initiated by maternal factors whereas labor at term may result from a culmination of both maternal and fetal endocrine signals in the mouse. Determining the similarities between term and preterm labor and further characterizing the mouse as a model for parturition leads to a better understanding of mouse parturition and improves its usefulness as a model.

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CHAPTER 3. Enzyme Immunoassay Experimental Protocol and Assay Validation

3.1 Introduction

The second study (Chapter 4) in this investigation encompassed the development of a working enzyme immunoassay (EIA) protocol for the laboratory. The details of the experimental protocol and assay validations will be outlined in this section.

3.2 Sample Preparation

3.2.1 Extraction

Prostaglandins were extracted from fetal membrane and placental tissue for measurement by EIA. The protocol was altered from existing in-lab radioimmunoassay (RIA) extraction procedures in combination with the protocol which accompanied the PGE_2 and $PGF_{2\alpha}$ EIA kits (Cayman Chemical Company, Ann Arbor, MI). The EIA is highly sensitive, therefore, all solutions were made with ultra pure Milli-Q water. Samples were kept on ice throughout the extraction procedure.

Tissue was pulverized in liquid nitrogen and 0.05 g of the tissue was homogenized in 500 µl of 100% ethanol in glass test tubes. Two blanks containing only ethanol were homogenized as well to ensure that the presence of tissue did not affect recovery values. Prior to homogenization, 2500 cpm of ^{3}H-PGE_2 was added to each test tube for recovery calculations (see below). Following homogenization, 4 ml of 50 mM citrate buffer (pH 3.5) were added to each sample to reduce the ethanol concentration to below 15% (necessary for purification of the sample). The samples were then inverted several times, placed at room temperature for 5 min and centrifuged at 12,000 *x g* for 25 min. Ninety percent of the supernatant was withdrawn for sample purification. This 10% loss was accounted for in recovery calculations (see below). The tubes were then inverted on an absorbent surface to remove any residual supernatant. The remaining protein pellet was capped, stored at 4°C and assayed on a 96 well plate within 24 h using the Micro BCA

Protein Assay Reagent Kit (Pierce, Rockford, IL). The basis of protein measurement by this method can be summarized in the following reaction scheme:

A.) Protein (peptide bonds) + Cu^{2+} ⟶ tetradentate-Cu^{1+} complex

B.) Cu^{1+} complex + BCA ⟶ BCA-Cu^{1+} complex (purple colored)

Interaction of Cu^{2+} with protein in an alkaline environment results in the reduction of this molecule to Cu^{1+}. Bicinchoninic acid (BCA) is used as the detection reagent for Cu^{1+}. The purple colored product of this reaction is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{1+}) and exhibits a strong absorbance at 562nm. Protein measurements were used to normalize PG levels which were expressed as pg/mg protein (see calculations below).

3.2.2 Purification

Samples were purified according to an altered version of the protocol which accompanied the PGE_2/$PGF_{2\alpha}$ EIA kits. Briefly, C-18 solid phase extraction (SPE) Sep-Pak cartridges were attached to 5 ml syringes and activated with methanol followed by 5 ml milli-Q water. The sample (90% of the supernatant above) was passed slowly through the column. The column was then washed again with milli-Q water followed by hexane. Finally, the PGs were eluted with ethyl acetate containing 1% methanol. The final volume in each tube was 5 ml. The ethyl acetate was evaporated to dryness under a gentle stream of nitrogen. Each sample was reconstituted with vortexing in 450 µl EIA buffer. Fifty microlitres of the sample were removed for scintillation counting (this was also accounted for in the final calculations – see below). The remaining sample (400 µl) was alliquotted and stored at -80°C until assayed.

3.3 Assay Protocol

3.3.1 General

Prostaglandin E_2 and $F_{2\alpha}$ levels were quantitated using an EIA kit specific to each prostaglandin. The EIAs were performed on a 96 well plate. Each well was pre-coated with secondary antibody (mouse anti-rabbit for $PGF_{2\alpha}$ and goat anti-mouse for PGE_2) and blocking proteins. This assay is based on the competition between the prostaglandins contained in a sample/standard and a synthetic prostaglandin conjugated to an

acetylcholinesterase (AChE) tracer for a limited number of primary antibody binding sites ($PGF_{2\alpha}$-specific rabbit anti-mouse IgG and PGE_2-specific mouse anti-mouse). The antibody-PG (sample/standard or tracer) complex binds to the secondary antibody site previously attached to the well. Since the amount of tracer is held constant while the sample concentration varies, the amount of PG in a sample is inversely proportional to the amount of tracer in the well (Figure 3-1).

Following incubation for 18 h at 4°C, the wells were washed to remove any unbound PG-antibody complexes. Ellman's reagent then was added to each well. Ellman's reagent measures AChE activity. It consists of acetylthiocholine and 5,5'-dithio-*bis*-(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by AChE produces thiocholine. Thiocholine reacts with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) to produce 5-thio-2-nitrobenzoic acid. This compound gives the solution a yellow color with a strong absorbance at 412 nm. The intensity of this color is proportional to the amount of tracer bound to the well and inversely proportional to the amount of sample PG in the well during the incubation.

3.3.2 Controls

Each sample was measured in three dilutions in duplicate. A standard curve and four controls were assayed in duplicate for each assay. The four control wells were assayed as follows: (1) Total Activity (TA) - total possible enzyme activity (maximum absorbance of the tracer), (2) Total Bound (B_o) – total possible binding (tracer and antibody only), (3) Non-Specific Binding (NSB) – non-specific (non-immunological) binding of the tracer to the well (tracer only) and (4) Blank (BL) – background absorbance of the Ellman's reagent. The tracer was not added to the TA well until after incubation and washing. Ellman's reagent was added to the blank well after incubation and washing. These controls were used to calculate final results taking into account background absorbance and binding.

3.3.3 Limits and Specificity

The detection limits for the PGE_2 and $PGF_{2\alpha}$ EIAs are 0.008 pg/µl and 0.015 pg/µl, respectively, as indicated by the supplier. The PGE_2 assay exhibits 100% specificity for PGE_2 and <0.01% specificity for $PGF_{2\alpha}$. The $PGF_{2\alpha}$ assay exhibits 100% specificity for

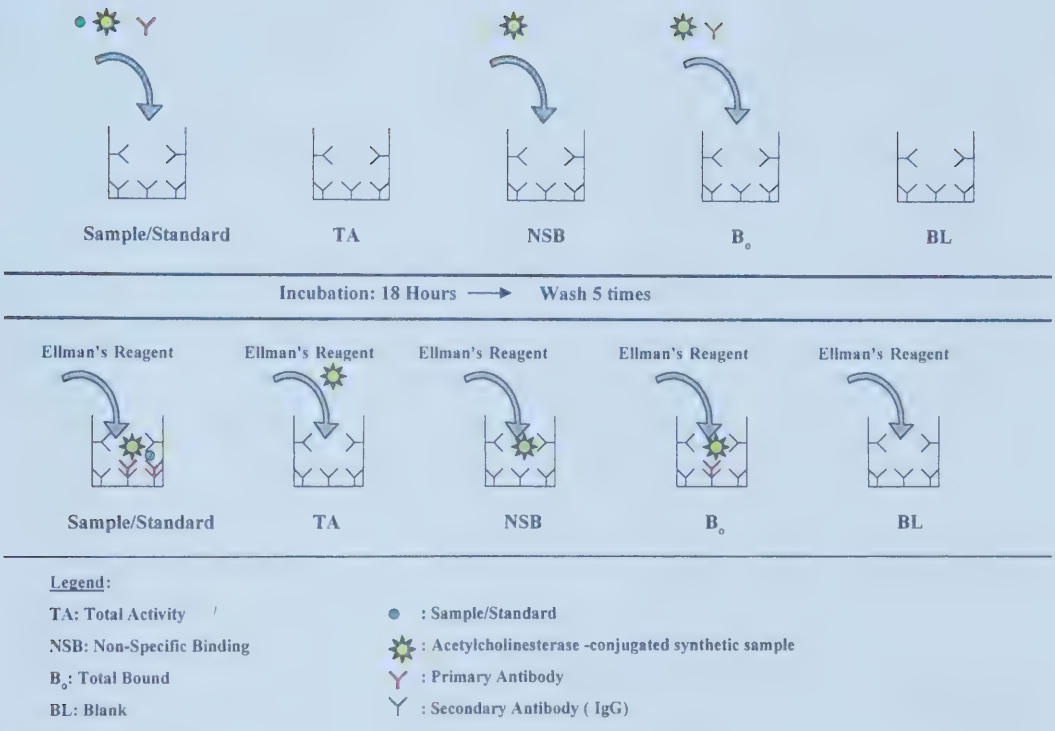

Figure 3-1 Enzyme Immunoassay Experimental Protocol

$PGF_{2\alpha}$ and 0.4% specificity for PGE_2.

3.4 Calculation of Results

3.4.1 Standard Curve

All values were calculated against a standard curve of known concentrations (pg/µl). Each point on the standard curve was expressed as percent of the maximum binding (%B/B_o), where B is the sample absorbance (bound) and B_o is the maximum binding. The following formula was used to calculate sample and standard %B/B_o:

$$\% B/B_o = \frac{B - NSB}{B_o - NSB} \times 100$$

The standard curve was linear between 20% and 80% B/B_o. Any values that fell outside this range were discarded. As well, a disparity greater than 20% between dilutions indicated interference, or poor pipetting technique. Any values that fell into this category were discarded or the sample was re-purified and re-assayed.

3.4.2 Calculation of Final Concentrations

As mentioned above, the final concentration of PGs in a given sample were expressed as pg/mg protein. The recovery factor is a measure of the effectiveness of the extraction procedure and was calculated for each individual sample. This factor was adjusted to account for the loss of 10% of the supernatant in the extraction procedure. Taking this into consideration the following formulae were used to calculate the concentration of PGs in a sample:

Recovery Factor:
$$\frac{\text{Initial Sample Volume}}{\text{Recovery Sample Volume}} \times \frac{\text{Final cpm - background cpm}}{\text{Initial cpm - background cpm}} \times 100$$

Adjusted Recovery Factor (adjusted for sample loss):
$$\frac{\text{Recovery Factor}}{0.90}$$

Final Concentration (pg/mg protein):

$$\frac{\text{sample (pg/µl) x Total Initial Sample Volume (µl)}}{\text{Adjusted Recovery Factor}}$$

$$\text{mg protein}$$

3.5 Assay Precision and Accuracy

3.5.1 Inter and Intra-assay Coefficients of Variation (COV).

Extracted samples were assayed in both the $PGF_{2\alpha}$ and PGE_2 EIAs to determine the variation between assays (inter-assay COV) and the variation between wells within one assay (intra-assay COV). The intra- and inter-assay COV for the PGE_2 EIA (n=5) were 10% and 14%, respectively. The intra- and inter-assay COV for the $PGF_{2\alpha}$ EIA (n=5) were 9% and 8%, respectively.

The following formula was used to calculate these measures of precision:

$$\frac{\text{(SD of replicates of sample)}}{\text{(Mean of replicates of samples)}} \times 100$$

3.5.2 Detecting Real Differences

Increasing concentrations of PG (spikes) were added to a sample and a blank in order to determine if both the PGE_2 and the $PGF_{2\alpha}$ EIAs could detect real changes in PG concentrations and to determine whether the extraction procedure interfered with this. The results of this test are shown in Figures 3-2 and 3-3. The amounts of PGE_2 added to the assay were 2pg, 5pg, and 10pg. The amounts of $PGF_{2\alpha}$ added were 1 pg, 2.5 pg, and 5 pg. The difference between the spike amounts for the two assays is due to the fact that the $PGF_{2\alpha}$ standard curve is half that of PGE_2. The results are expressed in total PGs (pg).

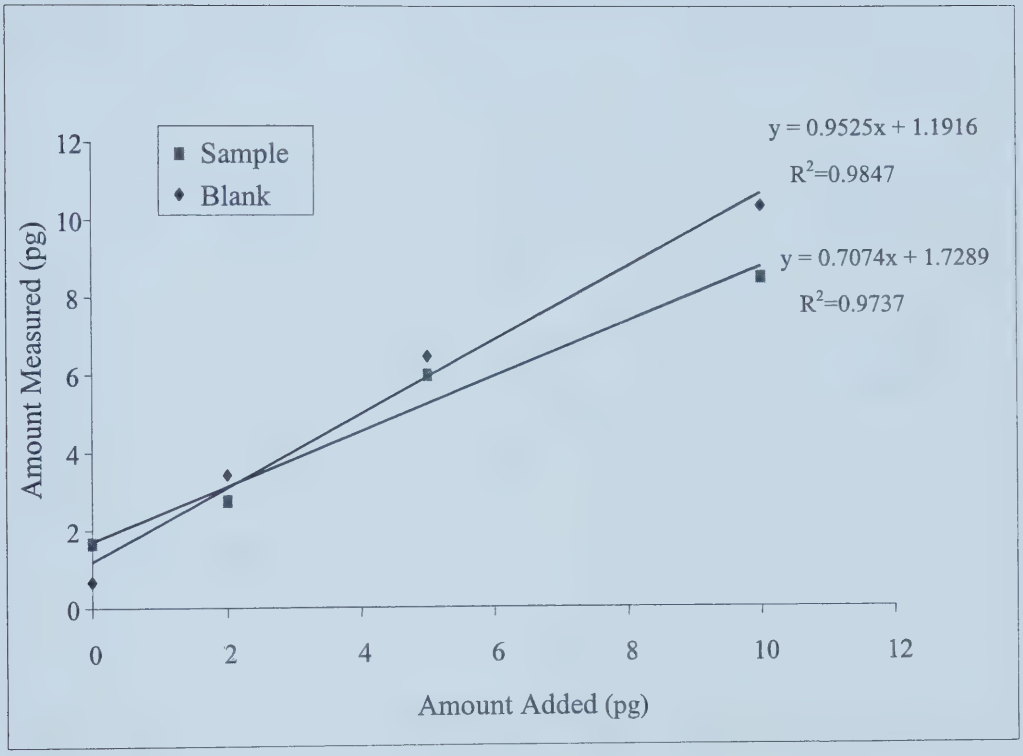

Figure 3-2 **PGE_2 assay accuracy.**

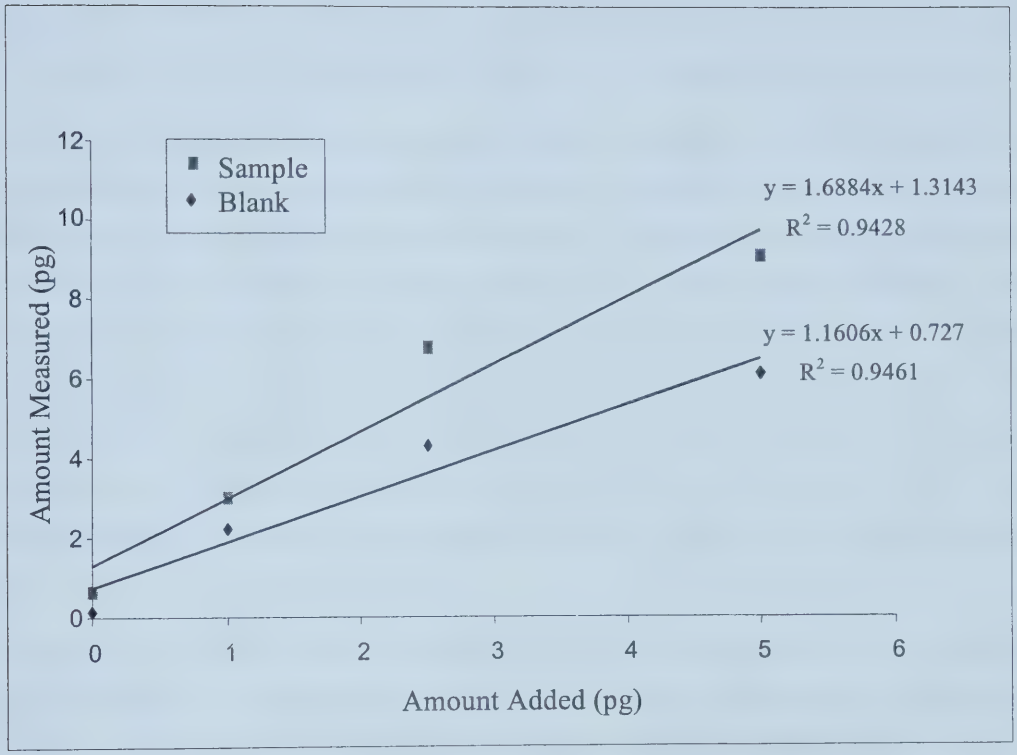

Figure 3-3 $PGF_{2\alpha}$ **assay accuracy**

3.6 Discussion

The EIA was chosen in this study over the RIA. Validations for both PGE_2 and $PGF_{2\alpha}$ RIAs were performed previously in our laboratory. In that study, the sensitivity limit of the RIA for both PGE_2 and $PGF_{2\alpha}$ was 0.1pg/ul. Therefore, the RIA is less sensitive than the EIA. In addition, the intra- and inter-assay COVs for PGE_2 were 3% and 12%, respectively. The intra- and inter-assay coefficients of variation for $PGF_{2\alpha}$ were 11% and 13%, respectively (1). These values were similar to those reported in the present study.

In addition, in the present study, both PG assays were able to detect changes in PG concentrations in both the sample and the blank. The slopes of the sample and blank lines were approximately equal for both assays. This indicates that the extraction procedure did not affect the ability of the assay to detect real differences. The intersection of the lines in the PGE_2 graph (Figure 3-2) may be due to the fact that both the sample and blank starting concentrations read at approximately the same point on the standard curve. Despite this, the slopes of the two lines were approximately equal. Validations of the RIA indicated similar results in that the assay could detect increasing concentrations of PG added to sample and blank and the slopes of the lines were similar (1).

In conclusion, the EIA would be a preferred method of PG measurement for 3 reasons: 1) the availability of an anti-body for both PGE_2 and $PGF_{2\alpha}$, 2) the high sensitivity and precision of the assay, 3) the assay is non-radioactive and thus a safer method.

LITERATURE CITED

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CHAPTER 4. The Effect of Maternal Progesterone Supplementation on Fetal Membrane and Placental PG Levels in the Mouse in Late Gestation and at Term Labor.

4.1 Introduction

Precise endocrine communication *in utero* in late gestation leads to the coordination of the events involved in labor in many species. Variation in this balance of hormonal cues can result in pre- or post-term delivery and increased risk of neonatal complications. Therefore, elucidating the mechanisms involved in the control of intrauterine hormonal levels is very important. Prostaglandins (PGs) have been shown to be key regulators of labor initiation in many species and are produced within the uterus by the fetal membranes and placenta (1-7). The level of PGs within the uterine compartment is an important determinant of the timing of labor onset (8-13).

PGs are synthesized from AA which is liberated from the *sn*-2 position of glycerophospholipids by phospholipase A_2 (PLA_2). AA is then metabolized into two inactive endoperoxide intermediates, PGG_2 and PGH_2, through the action of prostaglandin H-synthase (PGHS). PGHS exists as two isoforms PGHS-1 and PGHS-2. PGH_2 is converted to biologically active PGs by specific PG synthase/reductase enzymes (14). Biologically active PGs are converted to inactive metabolites by prostaglandin dehydrogenase (PGDH) (15-18). Each step in the PG biosynthetic pathway may be regulated. Progesterone (P_4), an important mediator of labor initiation, has been shown to regulate PG synthesis at the PLA_2, PGHS (19-24) and PGDH (25-27) points of the PG synthetic pathway both *in vitro* (28) and *in vivo* (29).

Two products of the arachidonic acid cascade, PGE_2 and $PGF_{2\alpha}$, increase in fetal membranes (29-31) placenta (31) and in the amniotic fluid and maternal plasma (32-35) around the time of labor. In both humans and sheep, late gestational changes in placental PG levels are regulated by fetal endocrine signals. In humans and other non-human primates, the androgen precursor 16OH-DHEA-S, provided by the fetus, precipitates

changes in the steroid milieu of the intrauterine environment. Similarly, in the sheep, cortisol from the fetal adrenal stimulates estrogen production by the placenta leading to increased PGE_2 production. In both species, steroid changes lead to increased PG synthesis (reviewed in Challis (1989), Olson (1995), Mijovic (1996) and Challis (2000) (8, 36-38)). In the mouse, a decrease in plasma P_4 results in the upregulation of factors involved in labor such as FP receptor, OTR, Cx-43, PGHS-2, $PGF_{2\alpha}$ and PGE_2 (24, 39, 40). As well, preterm labor can be induced in mice by ovariectomy (40) or treatment with RU486 (41) and ethanol-induced preterm labor can be inhibited by pretreatment with P_4 (24). Labor is delayed in FP and PGHS-1 knockout mice because the signal for luteolysis ($PGF_{2\alpha}$) is disrupted and there is no reduction in plasma P_4 levels (42, 43). Overall, plasma P_4 levels are an important regulator of labor in the mouse.

The mouse is a useful tool for the study of labor regulation due to its short gestation (approx. 19 days), availability of applicable molecular techniques and genetic manipulation experiments. In our mouse model of parturition we know that plasma P_4 levels decrease and uterine PGHS-2 mRNA expression and $PGF_{2\alpha}$ and PGE_2 levels increase at term labor, however, it is unknown whether PG levels increase in fetal membranes and placenta in late gestational and in correlation with labor onset. It is also unknown whether prostaglandins produced by the fetal membranes and placenta are affected by the decrease in plasma P_4. Therefore, this study was designed to investigate the profile of and the effect of maternal progesterone on fetal membrane and placenta PGE_2 and $PGF_{2\alpha}$ levels in late gestation, at the time of term labor and at post-term delivery.

4.2 Materials and Methods

4.2.1 Breeding

Nulliparous 10-12 week old female C57BL/6J mice (The Jackson Laboratories, Bar Harbor, ME) were bred for these experiments. These animals were housed in a temperature and humidity-controlled room, with a daily 12 h light, 12 h dark cycle (lights on at 6:00 am). One to four females were placed in each cage containing one male for breeding daily between 9:00 am and 11:00 am. After this time, females were examined for the presence of a seminal plug. Successfully bred females were weighed, housed

individually and then weighed daily after GD 10 in order to monitor the dams for pregnancy progression. The day on which the seminal plug was present was considered day 0 of gestation. The gestational length of this strain is 19.3 ± 0.01 days (24).

4.2.2 Treatment Regimen

Pregnant dams were separated into three treatment groups as follows: 1) untreated (n=6/timepoint), 2) placebo (n=4), 3) progesterone (n=6/timepoint;except post-term where n=4). The placebo treatment was only given to 4 individual dams for comparison with the untreated group in order show no effect of the anesthesia and drug administration. All placebo treated dams went into labor at term. Previous studies in our laboratory have also shown that the time of labor in placebo treated dams did not differ from untreated control (39). Three week release progesterone and placebo for progesterone pellets (2.5 mg/pellet, 21 day release, Innovative Research of America, Sarasota, FL) were administered on gestational day 15 (after which plasma progesterone concentrations fall in the untreated dams) (24). Pellets were given subcutaneously under general anesthesia (metofane). Once the dams were brought to a stage of surgical anesthesia, determined by toe-pad reflex, the pellet was administered and the animals were monitored until fully recovered. In these P_4 supplemented dams, P_4 levels do not reach levels conducive to labor onset until GD 20.4 ± 0.4 (40).

4.2.3 Tissue Collection

Fetal membranes and placentas were collected from pregnant mice on GD16, 17, 18, 19.3 (term labor) and 20.4 ± 0.4 (delayed labor onset – progesterone treated dams) (40). Tissue collection took place at 9:00 am on GD 16, 17 and 18 and after the delivery of the first pup at labor (term and post term). Tissue was also collected from progesterone treated dams at 9:00 am on GD 19 (time of normal term labor).

Pregnant dams were killed by cervical dislocation. The abdomen was opened and the uterus was carefully dissected and washed in a physiological (0.9%) saline solution. The uterus was then cut open taking care not to break the membrane sacs. Each placenta was gently detached from the uterine wall, the sac was broken and the membranes and placenta were removed from the fetus. The umbilical cord was cut close to the placenta to ensure that only placenta tissue was collected. The membranes were removed at the

interface between the fetal membranes and placenta. All tissue samples were rinsed in a physiological saline solution and excess solution was removed. The tissues were snap frozen in liquid nitrogen and stored at –70 °C until assayed.

4.2.4 Prostaglandin Extraction

Total prostaglandins were extracted from fetal membrane and placenta tissue for measurement by enzyme immunoassay (EIA). A complete outline of the protocol used for this procedure has been discussed in Chapter 3. Briefly, samples were kept on ice throughout the extraction procedure. Briefly, tissue was pulverized in liquid nitrogen and 0.05 g of the tissue was homogenized in 500 µl of 100% ethanol in glass test tubes. Two blanks containing only ethanol were homogenized as well to ensure that the presence of tissue did not affect recovery values. Prior to homogenization, 2500 cpm of ^{3}H-PGE_2 was added to each test tube for recovery calculations. Following homogenization, 4 ml of 50 mM citrate buffer (pH 3.5) was added to each sample to bring the ethanol concentration to under 15% (necessary for purification of the sample). The samples were then inverted several times, placed at room temperature for 5 min and centrifuged at 12,000 *x g* for 25 min. Ninety percent of the supernatant was withdrawn for sample purification. The tubes were then inverted on an absorbent surface to remove any residual supernatant. The remaining protein pellet was capped, stored at 4°C and assayed on a 96 well plate within 24 h using the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). All values were normalized to total protein and are expressed as pg prostaglandin/mg protein ± SEM.

4.2.5 Sample Purification

Samples were purified according to a protocol altered from that outline in the PGE_2/$PGF_{2\alpha}$ EIA kits (Cayman Chemical Company, Ann Arbor, MI). Briefly, C-18 solid phase extraction (SPE) Sep-Pak cartridges were attached to 5 ml syringes and activated with methanol followed by 5 ml milli-Q water. The sample (90% of the supernatant above) was passed slowly through the column. The column was then washed again with milli-Q water and hexane. Finally, the PGs were eluted with ethyl acetate containing 1% methanol. The ethyl acetate was then evaporated under a gentle stream of nitrogen. Each sample was reconstituted in 450 µl EIA buffer. Fifty microlitres of the

sample were removed for scintillation counting. The remaining sample was alliquotted (30 µl) and stored at –80 °C until the assay.

4.2.6 Prostaglandin Measurement

PGE_2 and $PGF_{2\alpha}$ were quantitated using an EIA kit specific to each PG. Absorbance values were measured at 412 nm. The intra and inter-assay COV for the PGE_2 EIA (n=5) were 10% and 14%, respectively. The intra and inter-assay COV for the $PGF_{2\alpha}$ EIA (n=5) were 9% and 8%, respectively.

4.2.7 Statistical Analysis

Statistical significance of the changes in PG levels in the untreated group was determined by one-way ANOVA. The effect of progesterone treatment on tissue PG levels was determined by two-way ANOVA with post-hoc analysis by Tukey's test. Within the progesterone treated group, significance of the measurements taken at post-term delivery was measured by one-way ANOVA. The difference between the two labor groups (term and post-term) was measured by Student's t-test. Significance was achieved when $p<0.05$.

4.3 RESULTS

4.3.1 Fetal Membrane PGE_2 and $PGF_{2\alpha}$

Figure 4-1 illustrates the effect of gestational age and P_4 treatment on fetal membrane PGE_2 levels. In the untreated controls, prostaglandin E_2 levels on GD 16 (998 ± 185 pg/mg protein) did not differ significantly from PGE_2 levels at term labor (607 ± 164 pg/mg protein). However, a significant reduction in PGE_2 levels was observed on GD 18 (189 ± 31 pg/mg protein). This value differed significantly from values at all other timepoints measured. A similar profile was observed in the P_4 treated tissue. There was no significant effect of treatment on fetal membrane PGE_2 levels in late gestation or at term labor.

Figure 4-2 illustrates the effect of gestational age and P_4 treatment on fetal membrane $PGF_{2\alpha}$ levels. Similar to fetal membrane PGE_2 measurements, $PGF_{2\alpha}$ levels on GD 16 (860 ± 114 pg/mg protein) did not differ significantly from levels at term labor (1130 ± 351 pg/mg protein). However, a significant decrease was observed on GD 18 (147 ± 21

pg/mg protein). This value differed significantly from values at all other sampling timepoints. A similar profile was also observed in the P_4 treated tissue. There was no significant effect of treatment on fetal membrane $PGF_{2\alpha}$ levels in late gestation or at the time of term labor.

4.3.2 Placental PGE_2 and $PGF_{2\alpha}$

Figure 4-3 illustrates the effect of gestational age and P_4 treatment on placental PGE_2 levels. An increase in PGE_2 levels was observed between GD 16 (436 ± 99 pg/mg protein) and GD 17 (944 ± 143 pg/mg protein). Levels returned to GD 16 values by GD 18 (471 ± 100 pg/mg protein) and these values were not different from term labor (730 ± 209 pg/mg protein). There was a significant effect of P_4 supplementation on placental PGE_2 levels on GD 18 ($p<0.05$) only. No effect of treatment was seen at other timepoints.

Figure 4-4 illustrates the effect of gestational age and P_4 treatment on placental $PGF_{2\alpha}$ levels. There was a significant effect of both gestational age and treatment on placental $PGF_{2\alpha}$ levels. Prostaglandin $F_{2\alpha}$ levels increased steadily from GD 16 (101 ± 10 pg/mg protein), reaching a peak on GD 18 (866 ± 248 pg/mg protein) and dropping slightly to GD 17 levels (391 ± 60 pg/mg protein) by the time of term labor (419 ± 50 pg/mg protein). Placental $PGF_{2\alpha}$ levels at term labor were significantly greater than levels on GD 16. With P_4 treatment, $PGF_{2\alpha}$ levels did not increase with advancing gestational age.

In addition, P_4 supplementation resulted in a significant decrease in $PGF_{2\alpha}$ on GD 18 when levels reached a low of 29 ± 4 pg/mg protein. A 30 fold difference was observed between this value and levels observed in the untreated control group on GD 18 (866 ± 249 pg/mg protein). On GD 19.3, placental $PGF_{2\alpha}$ returned to GD 16 values in the P_4 treated group. This value was significantly lower than the control group in labor. Overall, there was a significant inhibitory effect of P_4 treatment on placental $PGF_{2\alpha}$ levels in late gestation and at the time of term labor.

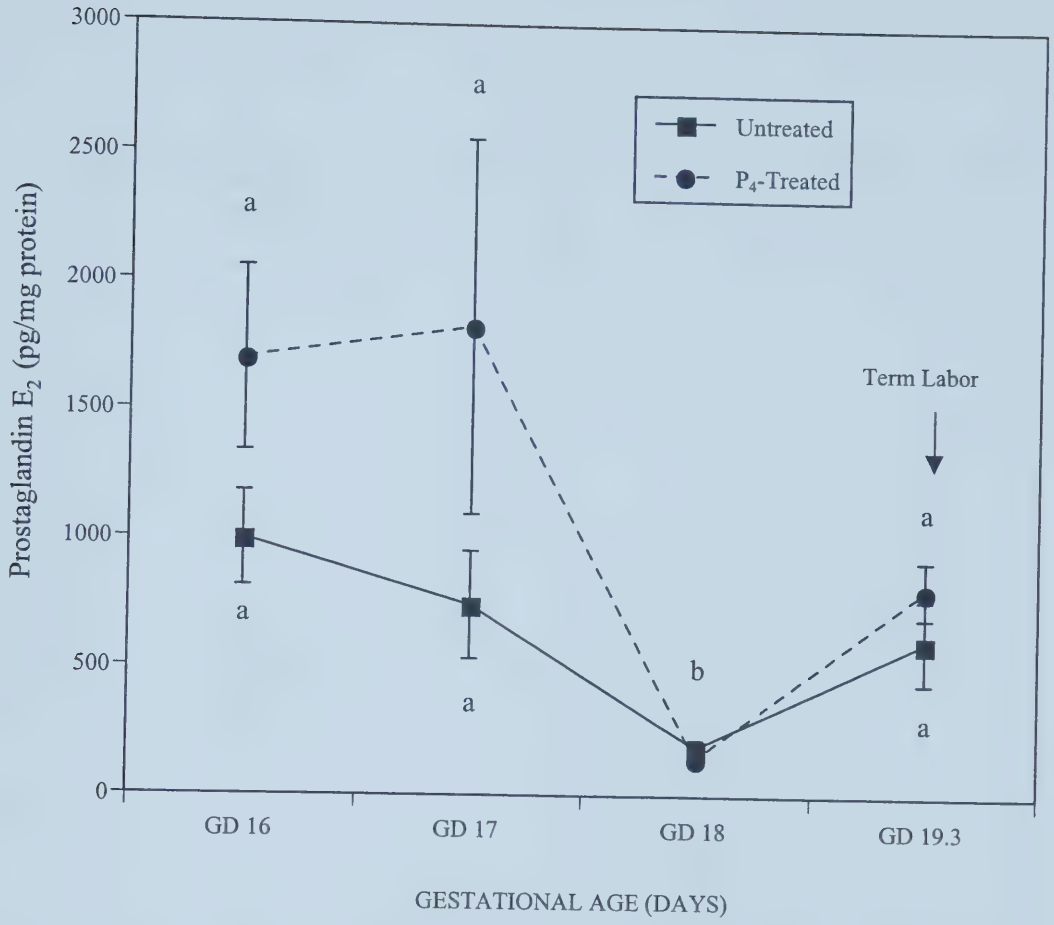

Figure 4-1 Fetal membrane PGE_2 levels from untreated and P_4-treated dams.

Prostaglandin levels were measured by EIA and are expressed as pg/mg protein (n=6/timepoint). In the fetal membranes, PGE_2 levels decreased at GD 18 in untreated tissue and were not affected by P_4 treatment. Statistical significance was determined by one- and two-way ANOVA ($p<0.05$) with post-hoc analysis by Tukey's test. Letters that differ indicate significance.

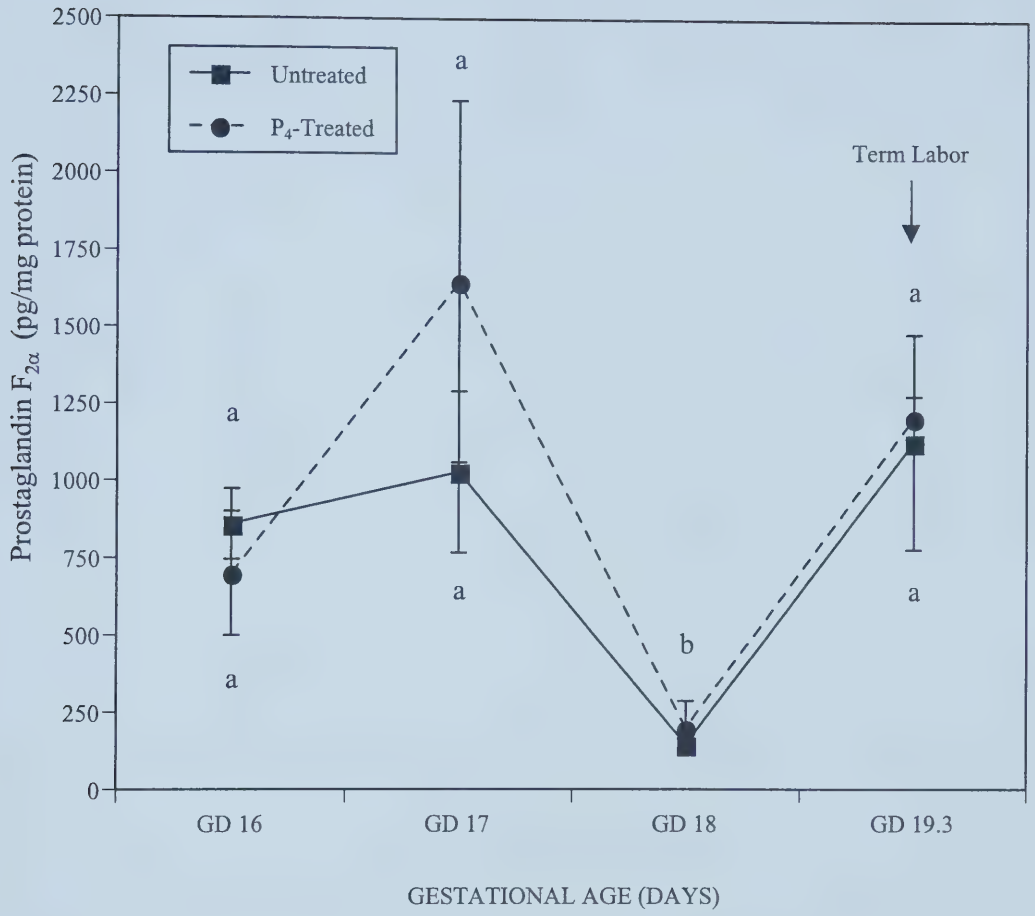

Figure 4-2 Fetal membrane $PGF_{2\alpha}$ levels from untreated and P_4-treated dams.

Prostaglandin levels were measured by EIA and are expressed as pg/mg protein (n=6/timepoint). In the fetal membranes, $PGF_{2\alpha}$ levels decreased significantly at GD 18 in untreated tissue and were not affected by P_4 treatment. Statistical significance was determined by one- and two-way ANOVA ($p<0.05$) with post-hoc analysis by Tukey's test. Letters that differ indicate significance.

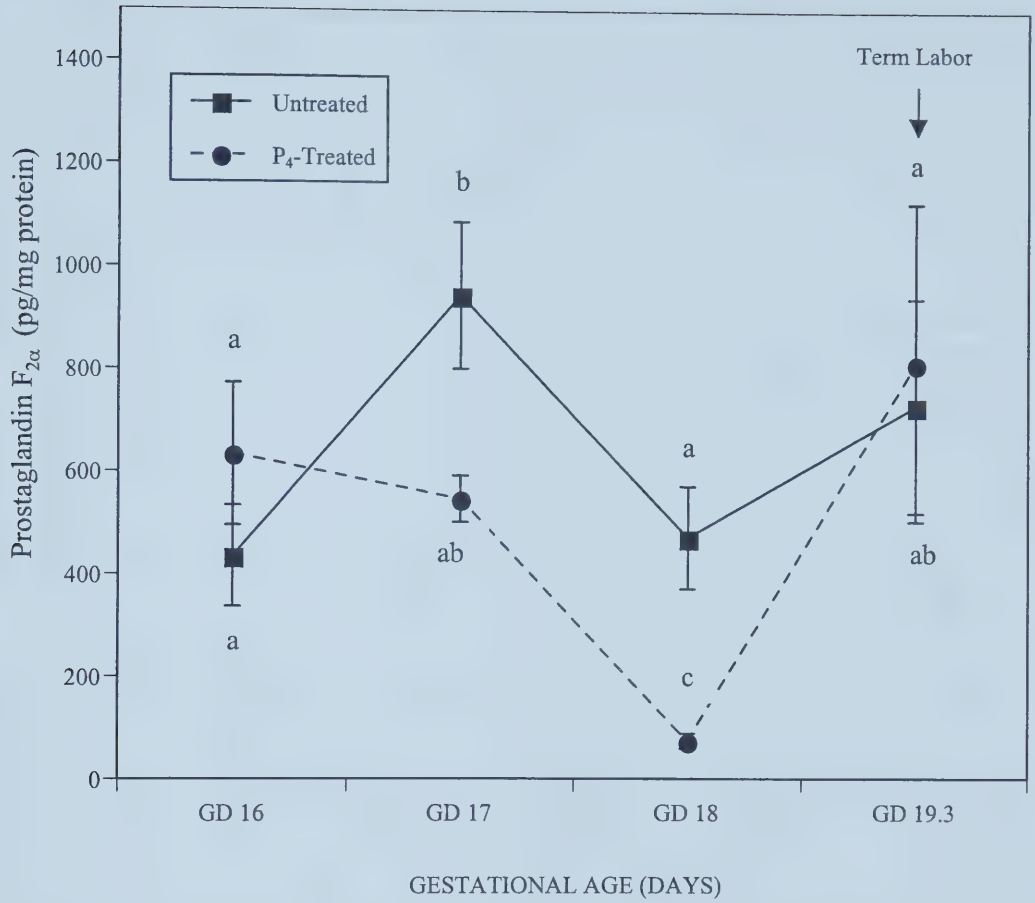

Figure 4-3 Placental PGE_2 levels from untreated and P_4-treated dams

Prostaglandin levels were measured by EIA and are expressed as pg/mg protein (n=6/timepoint). In the placenta, PGE_2 levels fluctuated with gestational age but did not differ between GD 16 and GD 19.3. These levels were diminished by P_4 treatment on GD 18 only. Statistical significance was determined by one- and two-way ANOVA ($p<0.05$) with post-hoc analysis by Tukey's test. Letters that differ indicate significance.

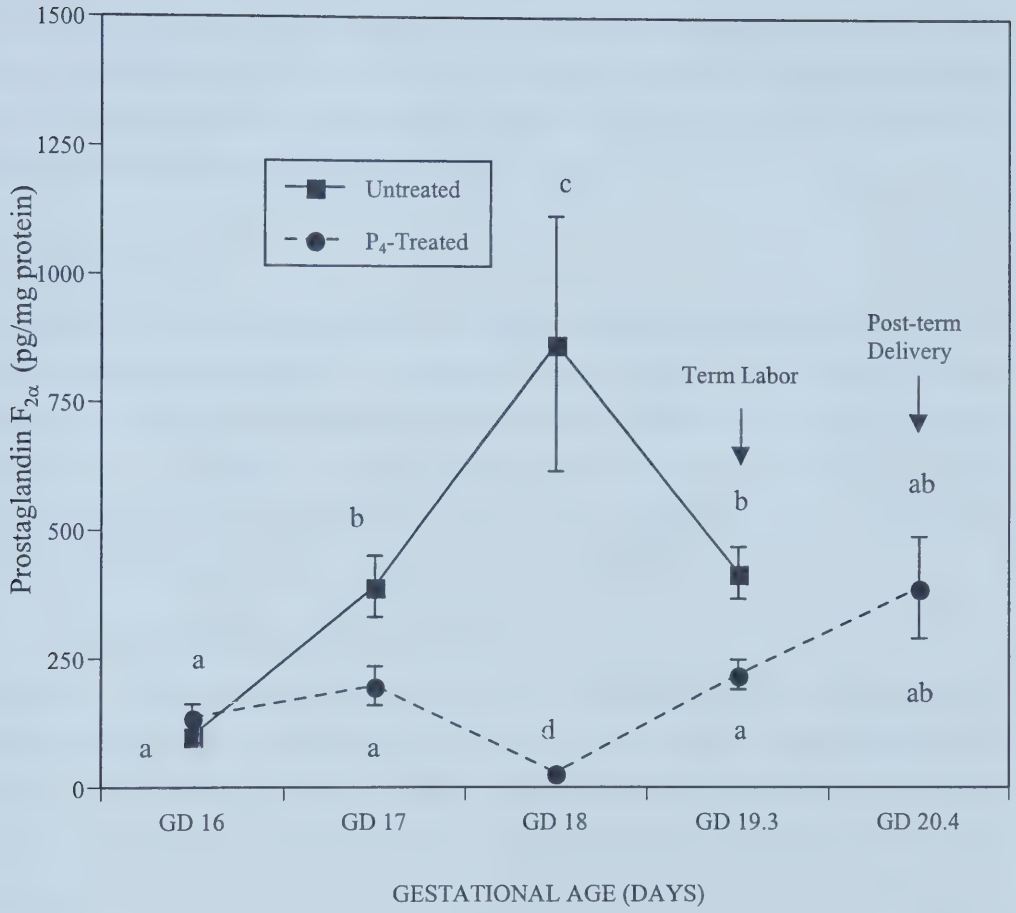

Figure 4-4 Placental $PGF_{2\alpha}$ levels from untreated and P_4-treated dams

Prostaglandin levels were measured by EIA and are expressed as pg/mg protein (n=6/timepoint). In the placenta, $PGF_{2\alpha}$ levels increased steadily between GD 16 and GD 18 and decreased slightly at term in untreated tissue. Levels were inhibited at all timepoints by P_4 treatment and increased towards term levels at post-term labor. Statistical significance was determined by one- and two-way ANOVA ($p<0.05$) with post-hoc analysis by Tukey's test. Letters that differ indicate significance.

4.3.3 Placental $PGF_{2\alpha}$ at Post-term Delivery

To further substantiate the effect of P_4 treatment on placental $PGF_{2\alpha}$ levels, placentas were collected from an additional group of P_4 treated dams that entered labor on GD 20.4 ± 0.4 (delayed delivery) (40). Placental $PGF_{2\alpha}$ levels increased between GD 19.3 (220 ± 29 pg/mg protein) and GD 20.4 ± 0.4 (390 ± 99 pg/mg protein), although this increase was not significant ($p>0.05$). Importantly, values at GD 21 did not differ significantly from levels in the untreated control group at term labor.

4.4 DISCUSSION

The results of this study indicate that in the fetal membranes neither PGE_2 nor $PGF_{2\alpha}$ levels increase with term labor. In contrast, placental $PGF_{2\alpha}$ levels increased in late gestation and maternal progesterone supplementation inhibited the rise in placental $PGF_{2\alpha}$ levels. In addition, an interesting trend was observed on GD 18 in all measurements except in placental PGE_2 levels. Progesterone is known to regulate PGs within the intrauterine tissues around the time of labor (22, 24).

4.4.1 Prostaglandin Levels in Late Gestation

The profile of fetal membrane PGE_2 and $PGF_{2\alpha}$ and placental $PGF_{2\alpha}$ in late gestation is intriguing and not easily explained. On GD 18, a large reduction (approximately 4-6 times) in fetal membrane PGE_2 and $PGF_{2\alpha}$ levels was observed, while levels at all other timepoints were similar. This may be due to increased PGDH activity on GD 18. In humans, the chorion is a major site of PG metabolism (29, 44, 45). In rodents, the chorion is replace by the yolk sac which may exhibit increased PGDH activity on GD 18. However, since levels at all other timepoints, including term labor were similar, this may not be the case. PGs produced in murine fetal membranes may affect yolk sac blood flow, since PGs have been shown to regulate vascular tone (31, 46-48). The sharp reduction in PG levels observed may serve to elicit contractile or relaxant responses in the yolk sac vasculature, depending on the type of PG receptors present.

In the placenta, PGE_2 levels did not change significantly on GD 18 while $PGF_{2\alpha}$ levels increased by approximately 6 fold. These results indicate different regulatory control of each PG synthase enzyme within the placenta. In both fetal membranes and placenta, the most dramatic changes in PG levels were observed on GD 18. Progesterone metabolites

may play a role in this trend. It has been shown that 5α-reductase Type I (5α-R1) and 3α-hydroxysteroid dehydrogenase (3α-HSD) mRNA expression and activity peak on GD 18 in murine uterine tissue (49). The metabolism of P_4 by the sequential action of these enzymes leads to the production of allopregnenolone, a biologically active neurosteroid (50). Metabolism also has been reported in rat fetal membranes and placental tissue (51, 52). The dramatic changes in PG levels observed on GD 18 in untreated controls may be due to action of P_4 metabolites.

4.4.2 The Effect of Progesterone on Prostaglandin Levels

There was no effect of P_4 on the gestational profile of fetal membrane PGs. This may be due to a lack of P_4 receptors (PR) in this tissue. In the placenta, PGE_2 levels were inhibited by P_4 supplementation on GD 18 only. Placental $PGF_{2\alpha}$ levels, however, were inhibited by P_4 supplementation throughout most of late gestation and at term labor. Furthermore, in the supplemented dams, at post-term delivery, when P_4 decreased to a level sufficient to allow delivery (39), $PGF_{2\alpha}$ increased to levels near normal term labor values. Since the greatest inhibitory effect of P_4 was on placental $PGF_{2\alpha}$, not PGE_2, P_4 may inhibit PGF synthase. PGF synthase protein has been detected by Western Immunoblot in murine placenta (53). However, the gestational profile, regulation of PGF synthase is unknown. Further investigation is required in this area.

Interestingly, P_4 inhibited placental $PGF_{2\alpha}$ levels to the greatest extent on GD 18 (by approximately 30 fold). One possible explanation could be an increased expression of PR at that timepoint. Other studies have indicated that P_4 supplementation can maintain P_4 receptor in the decidua basalis of the rat placenta over and above levels observed in ovariectomized animals (54, 55). However, in the same study, PR was not detectable in the labyrinthine tissue of the rat placenta (56) and the placenta may serve as a barrier to P_4 and P_4 metabolites (57). In the present study, maternal and fetal components of the murine placenta were not measured separately and the effect seen may be decidual rather than fetal. Further localization experiments need to be performed in order to determine whether the effect observed was in the fetal or maternal portions of the tissues collected.

Progesterone has been shown to downregulate PLA_2 and PGHS activity. Evidence of P_4 suppression of PGHS-2 has been reported in bovine endometrial cells (20, 28),

myometrial myocytes (28) and cervix (21), in human endometrium (58) and in rat preovulatory follicles (23). Progesterone may also suppress mouse uterine PGHS-2 (22). In addition, P_4 agonists upregulate PG metabolism (PGDH) in chorionic trophoblast cells in culture (27), and RU486 (progesterone receptor antagonist) has been shown to decrease metabolism (25) and decrease PGDH activity (26) in decidual tissue and more specifically in decidual blood vessels (59).

4.4.3 Summary

Information from this study contributes to the growing body of information on the endocrinological events that occur prior to and during murine labor. These events are essential to elucidate since the mouse is emerging as an important model in genetic manipulation experiments. Although genetic manipulations provide useful information about the role of certain gene products, it is important to understand the endocrinological events that occur under normal genetic conditions. The results of the present study provide possible evidence of maternal P_4 control of placental (possibly fetal) $PGF_{2\alpha}$ levels.

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The purpose of this study was to further characterize PG production in a mouse model of parturition by determining the changes in PGHS-1 and PGHS-2 mRNA expression and PGE_2 and $PGF_{2\alpha}$ levels in murine fetal membranes and placenta in late gestation and with term labor. In addition, PGHS mRNA expression was measured at ethanol induced preterm labor and PG levels were measured after supplementation with P_4.

Previous studies in our laboratory have shown that PGHS-2 mRNA expression as well as $PGF_{2\alpha}$ and PGE_2 levels increase in the uterus at the time of term labor (1, 2). In the present study, fetal membrane PGHS-1 and PGHS-2 mRNA expression increased late gestation and at the time of term labor while neither PGE_2 or $PGF_{2\alpha}$ levels increased (figure 5-1). This may indicate that either PGHS mRNA expression is not translated into PGHS protein or that PGHS enzyme activity does not increase in correspondence with the increased mRNA expression measured. Furthermore, although the levels of PGs did not increase, they may exert biological activity through increased receptor availability. In the placenta, PGHS-2 mRNA expression and $PGF_{2\alpha}$ levels did increase around the time of term labor. Since the pattern of PGHS expression and PG levels in the placenta at term labor closely mimic the pattern observed in the uterus, placental $PGF_{2\alpha}$ may be an important regulator of myometrial contractility at term labor.

Placental PGHS-2 mRNA expression increased steadily after GD 16 while no increase was observed in PGHS-1 mRNA expression throughout late gestation. PGE_2 levels increased in coordination with PGHS-2 expression between GD 16 and 17, but, returned to GD 16 levels by GD 18. $PGF_{2\alpha}$ levels increased between GD 16 and GD 18, and decreased slightly by the time of term labor. Thus, $PGF_{2\alpha}$ levels correlated with increasing PGHS-2 mRNA up to GD 18, after which PGHS-2 mRNA expression was maintained and $PGF_{2\alpha}$ levels decreased. This may be due to decreased PGF synthase activity after GD 18 despite continued PGHS-2 mRNA expression. Additionally, the sharp changes observed in PG levels on GD 18 in both the fetal membrane and placental tissues did not correlate with PGHS mRNA expression. This may also be due to

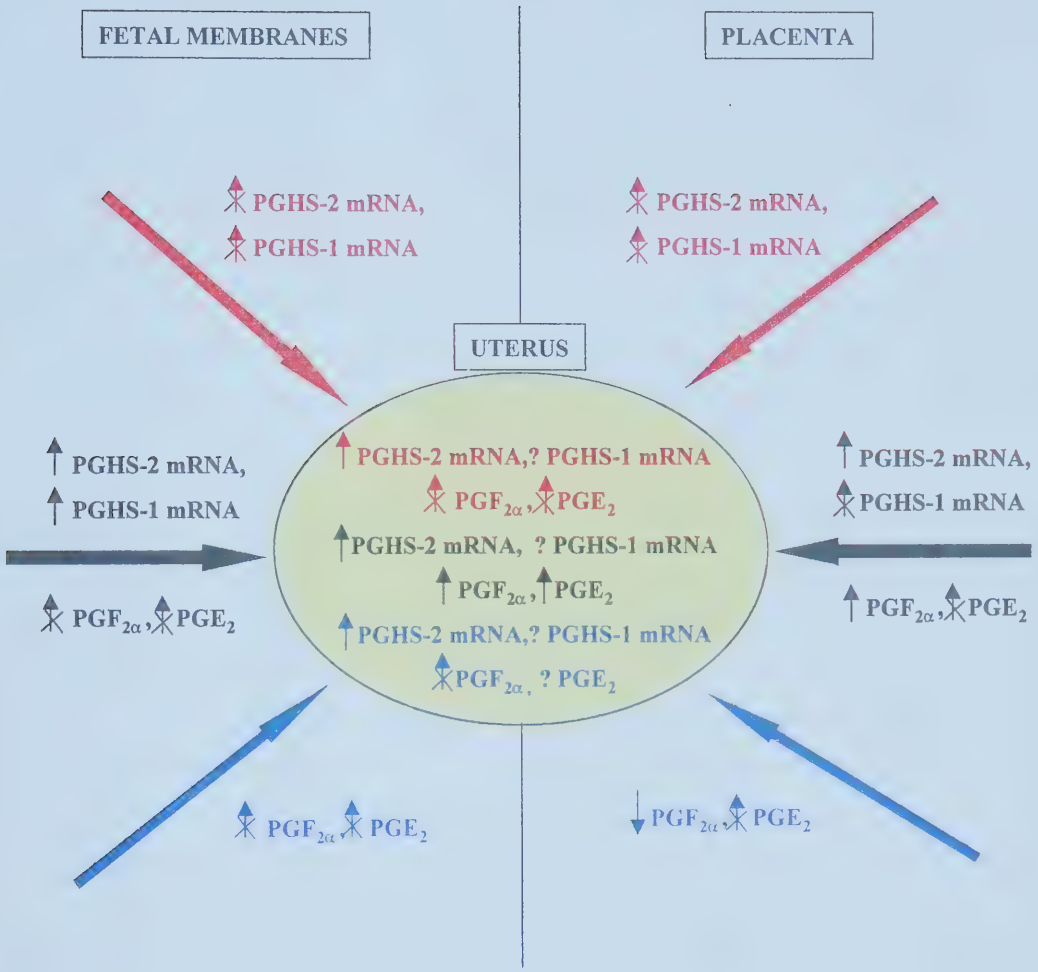

Figure 5-1 Uterine, Fetal Membrane and Placental PGs

The hypothesized contribution of uterine, fetal membrane and placental PGs to uterine PG levels at the time of ethanol induced preterm labor (red), term labor (black) and progesterone supplementation (delayed labor, blue) in the mouse. An X through an arrow indicates no increase.

regulation downstream in the PG biosynthetic pathway at the PG synthase level. Information on regulation and expression of PG synthase enzymes in late gestation has not been studied, although PGFS has been localized to murine placental tissue (3).

A previous study in our laboratory showed that during ethanol-induced preterm labor, uterine PGHS-2 mRNA expression increased (2). In the present study, neither PGHS-1 nor PGHS-2 mRNA expression increased at ethanol-induced preterm labor in the fetal membranes or placenta (both containing fetal tissue). These data, combined with the term labor data, suggest that 1) term and ethanol-induced preterm labor are regulated differently in the mouse and 2) that term labor may occur through a culmination of fetal and maternal endocrine signals, while preterm labor may be initiated by maternal factors alone.

Other studies have shown that ovine fetal cotyledon PGHS-2 mRNA expression increases prior to maternal (uterine) PGHS-2 mRNA expression in late gestation (4, 5), suggesting a fetal to maternal progression of PG synthesis prior to labor onset. In our murine model of parturition, placental PGHS-2 mRNA increased at GD 17.5 (Chapter 2) while uterine PGHS-2 mRNA expression increased later at term labor (1, 2). In addition, placental $PGF_{2\alpha}$ levels (Chapter 4) peak at GD 18, while a previous study in our laboratory indicated that uterine $PGF_{2\alpha}$ levels continued to rise and peaked with term labor (2). These results support a fetal to maternal progression of PG synthesis in late gestation.

Finally, the results of the P_4 supplementation suggest maternal suppression of placental $PGF_{2\alpha}$. This may occur through interaction with PGHS or PGFS (since the inhibitory effect of P_4 was not observed for PGE_2 in same placental samples). Previous studies in our laboratory and others have indicated that P_4 may suppress uterine PGHS-2 mRNA levels in the mouse (1, 6). Also, in support of a role for P_4 in the murine placenta, a recent study has shown that P_4 is an important regulator of protein synthesis in the rat placenta and ovariectomy or treatment with RU486 causes a decrease in DNA content (7). However, the same study suggests that this regulation takes place in the decidua basalis since PR has not been localized in the labyrinthine portion of the placenta (8). Rodent decidualization takes place primarily in the area around the site of placental development as opposed to the human decidua which is a continual layer around the entire fetus (9). Tissue collection procedures in the present study did not take measures

to dissect decidual components from placental tissue, therefore, the effect observed may have been decidual.

The contribution of the fetus to the signal for birth is not known in the mouse. Similar to other models, this study has shown that murine placental PGs do appear to be important around the time of labor since both the synthetic enzymes and PGs increase around the time of labor. However, unlike sheep and humans, placental PG levels may be controlled by maternal factors (P_4) rather than fetal factors. This would be practical since there are between 8-10 fetuses present in the uterus at one time and a fetal signal for labor would involve a coordination between multiple fetuses, in two separate uterine horns. As well, some fetuses may not be fully developed or partially resorbed and may cause disruption in the signal and prevent it from passing on to the next fetus. Finally, since the ratio of fetal:maternal weight in the pregnant mouse is much less than other species, it may be necessary that maternal factors regulate intrauterine PG synthesis in order to maintain maternal health.

As mentioned, the murine uterus is comprised of two uterine horns. Interestingly, each horn has a separate vasculature. Communication in utero may take place either trans-amniotically or via the uterine vasculature in which blood flows from the cervix to the ovary (10, 11). Studies have shown that female rats can be masculinized by male littermates located caudally *in utero* and that passage of androgen occurs within the vasculature (10, 11). In the present study P_4 may gain access to each placenta via the uterine artery. Thus, the decrease in plasma P_4 in late gestation may be a signal for increased PG synthesis by the placenta.

The predominant analysis used in this study was the one-way ANOVA. This statistical test analyzes differences between the means of each treatment group, which were, in this case, increasing gestational age. This test was appropriate because the data were analyzed to determine a profile of the outcome measure (enzyme expression or PG levels) at each timepoint, rather than a general trend for an increase or decrease (in which case Regression analysis would be used). Also, because it is difficult to determine the exact point at which the dam goes into labor, we cannot conclude that a change at GD 19.3 indicates that the factor is involved in labor initiation. Therefore, examining the

profile across late gestation gives us the ability to observe any changes prior to the GD 19.3 timepoint.

Most importantly, the information obtained in this study is not direct evidence of a role for fetal PGs in murine parturition, although the tissues examined are of fetal origin. Rather, this study has determined that, in the mouse, fetal membrane and placental PGs may be important in late gestation and at term labor, that placental PGs may be regulated by maternal P_4 in late gestation, and, that neither fetal membrane nor placental PGs appear to be important around the time ethanol-induced preterm labor. The PGs measured in this study may have important implications in other physiological processes occurring around the time of labor, other than labor itself. Such roles may include mediation of the inflammatory response, regulation of placental, uterine and/or yolk sac vascular tone, membrane rupture and cervical ripening in addition to roles in fetal circulation such as ductus arteriosus patency. Furthermore, although all PG levels did not increase at term labor, their ability to exhert biological activity may have increased through increased receptor availability. Finally, although PGHS mRNA expression did not change with ethanol-induced preterm labor, increased PG production may have been regulated at the translational level or by changes in enzyme activity.

The results from this study indicate the need for further investigation into the role and regulation of fetal PGs at term labor in the mouse. Future directions include the following:

- To determine the profile of and cellular localization of PGHS, PGFS and PR in placentas collected from P_4 supplemented dams
- To determine placental PGE_2 levels in the P_4 supplemented group at the time of post-term delivery
- To determine the profile of EP and FP receptors in fetal membrane and placental tissue in late gestation with term labor, ethanol-induced preterm labor, and post-term delivery
- To determine PG levels, PGHS protein levels and PGHS-specific activity in fetal membranes and placental tissue at ethanol-induced preterm labor

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